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Inheritance of virulence in the root rot pathogen *Phytophthora sojae*

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Graduate Program in Biology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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Inheritance of virulence in the root rot pathogen *Phytophthora sojae*

(Thesis format: Monograph)

by

Sirjana Devi Shrestha

Graduate Program in Biology

A thesis submitted in partial fulfillment of the
requirements for the degree of
Masters of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

The oomycete *Phytophthora sojae* causes stem and root rot of soybean plants. The interaction of pathogen avirulence (*Avr*) and host resistance (*R*)-genes determine the disease outcome. The *Avr3a* mRNA transcript level is variable among *P. sojae* strains and determines virulence towards the *R*-gene *Rps3a*. To study the inheritance of virulence, genetic crosses and self-fertilizations were performed. A cross between *P. sojae* strains ACR10 and P7076 causes transgenerational gene silencing of *Avr3a* allele, and this effect is meiotically stable up to the F₅ generation. However, test-crosses of F₁ (*Avr3a*^{ACR10}/*Avr3a*^{P7076}) with strain P6497 result in expression of *Avr3a* in all progeny and release of silencing of the *Avr3a*^{P7076} allele. Progeny from P6497 X ACR10 crosses showed unusual inheritance for *Avr3a* expression. Overall, we conclude that *Avr3a* gene silencing is strain specific and could rely on epistatic factors. This study will lead to a better understanding of infection and virulence mechanisms that will help to better manage and safeguard soybean production.

Key Words: *Phytophthora sojae*, soybean, virulence, avirulence, genetic crosses, oospore, *Avr3a* gene, transcript, RT-PCR, gene silencing, epistatic, gene conversion

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Table of Contents

Abstract	ii
Acknowledgements	iii
Table of Contents	iv
Lists of Tables	vii
List of Figures	viii
List of Abbreviations	xi
Chapter 1: Introduction	1
1.1. Biology of <i>Phytophthora sojae</i>	1
1.1.1. Evolutionary History	1
1.1.2. Life cycle of <i>P. sojae</i>	5
1.1.3. Pathogenesis.....	6
1.2. Economy	9
1.3. Control of <i>P. sojae</i>	9
1.4. Genomics of <i>P. sojae</i>	10
1.4.1. Genetic crosses and inheritance of <i>P. sojae</i>	11
1.4.2. Gene-for-gene interactions between <i>P. sojae</i> Avr genes and soybean <i>Rps</i> genes	11
1.4.3. Avr genes of <i>P. sojae</i>	13
1.5. Gain of virulence changes to <i>P. sojae</i> Avr genes	19
1.6. Thesis Hypotheses	21
1.7. Thesis Objectives	21

Chapter 2: Materials and Methods	23
2.1 <i>Phytophthora sojae</i> strains	23
2.2 Plant materials	23
2.3 Culture and cross of <i>P. sojae</i>	23
2.3.1 Generation of F ₄ and F ₅ progeny from <i>P. sojae</i> cross ACR10 X P7076	24
2.3.2 Test-crossing of <i>P. sojae</i> F ₁ (ACR10 X P7076) X P6497 (reference strain).	27
2.3.3 Crossing of <i>P. sojae</i> strains P6497 X ACR10	27
2.4 Isolation of oospores	27
2.5 Genomic DNA isolation	31
2.6 Polymerase chain reaction (PCR)	32
2.7 Hybrid determination	32
2.8 Restriction Digestion	33
2.9 Extraction of RNA and reverse transcriptase polymerase chain reaction (RT-PCR)	35
2.10 Virulence assay	36
2.11 Identification of maternal parent in <i>P. sojae</i> cross P6497 X ACR10	37
2.12 Quantitative real time PCR (qRT-PCR) to study the expression level of <i>Avr3a</i> gene among <i>P. sojae</i> hybrids	37
Chapter 3: Results	41
3.1 Transgenerational gene silencing of <i>Avr3a</i> is meiotically stable over multiple generations in progeny from a cross of ACR10 X P7076	41
3.2 Gene silencing of <i>Avr3a</i> is released by out-crossing	49

3.3	Release of silencing of <i>Avr3a</i> ^{P7076} in progeny from test-cross P6497 X F ₁ (ACR10 X P7076)	62
3.4	Crosses of <i>P. sojae</i> strains P6497 X ACR10 results in F ₁ hybrids that segregate for virulence towards <i>Rps3a</i>	64
3.5	Maternal or paternal effects do not determine gene silencing of <i>Avr3a</i> in F ₁ progeny from P6497 X ACR10	65
3.6	Self-fertilization of <i>P. sojae</i> strain P6497 and ACR10 produce true-breeding progeny for <i>Avr3a</i> virulence trait and gene expression	71
3.7	Segregation of virulence towards <i>Rps3a</i> , and <i>Avr3a</i> expression in F ₂ progeny from P6497 X ACR10.....	71
Chapter 4: Discussion		79
4.1	Stable transgenerational gene silencing of <i>Avr3a</i> in <i>P. sojae</i>	79
4.2	Test-cross of <i>P. sojae</i> strains results in release of gene silencing	82
4.3	Inheritance of <i>Avr3a</i> in a cross of <i>P. sojae</i> strains P6497 X ACR10.....	84
Chapter 5: Summary and Conclusion		88
References		90
CURRICULUM VITAE		103

Lists of Tables

Table 1.1 The <i>Avr3a</i> alleles in different strains of <i>P. sojae</i>	18
Table 2.1 Strains of <i>P. sojae</i> and their original sites	25
Table 2.2 Culture media used for <i>P. sojae</i>	25
Table 2.3 Oligonucleotide Primers used in this study	40
Table 3.1 Virulence outcomes and <i>Avr3a</i> transcript detection in F ₄ progeny	47
Table 3.2 Virulence outcomes and <i>Avr3a</i> transcript detection in F ₅ progeny	48
Table 3.3 Virulence outcomes and <i>Avr3a</i> transcript detection in progeny of P6497 X F ₁ - 62 (ACR10/P7076)	59
Table 3.4 Virulence outcomes and <i>Avr3a</i> transcript detection in progeny of P6497 X F ₁ - 81(ACR10/P7076)	60
Table 3.5 Scoring of virulence assay results of all F ₁ hybrids (P6497 X ACR10) and their respective <i>Avr3a</i> gene expression pattern	69
Table 3.6 Summary of results for virulence phenotype and <i>Avr3a</i> transcript analysis, for F ₂ populations developed from avirulent F ₁ (P6497 X ACR10)	78

List of Figures

Figure 1.1 Eukaryotic phylogeny based on morphology.....	3
Figure 1.2 Colony morphology of <i>P. sojae</i>	3
Figure 1.3 Oospore of <i>P. sojae</i>	4
Figure 1.4 Life cycle of <i>P. sojae</i>	8
Figure 1.5 <i>Avr3a</i> gene model of <i>P. sojae</i> strain P6497.....	16
Figure 1.6 Predicted protein sequences of three alleles of the <i>Avr3a</i> gene identified among <i>P. sojae</i>	17
Figure 2.1 <i>P. sojae</i> is homothallic diploid organism.....	26
Figure 2.2 Schematics of self-fertilization.....	28
Figure 2.3 Diagrammatic flow chart of crossing of <i>P. sojae</i> and isolation of oospores....	30
Figure 2.4 Restriction patterns of DNA markers used.....	34
Figure 2.5 Photograph showing inoculum of <i>P. sojae</i> in the soybean hypocotyl.....	39
Figure 3.1 Photographs of disease outcomes in soybean plants infected with <i>P. sojae</i> parental strains, F ₄ and F ₅ progeny.....	43
Figure 3.2 RT-PCR analyses of F ₄ and F ₅ progeny for the <i>Avr3a</i> mRNA transcript.....	45

Figure 3.3 DNA marker analysis to identify hybrid cultures from a cross of <i>P. sojae</i> P6497 X F ₁ (ACR10 X P7076).....	51
Figure 3.4 Deduced <i>Avr3a</i> genotypes of the progeny isolated from test cross of P6497 X F ₁ (ACR10 X P7076).....	55
Figure 3.5 Photographs illustrating the outcomes of virulence assays of hybrids isolated from test cross of P6497 X F ₁ (ACR10 X P7076).....	56
Figure 3.6 Analysis for <i>Avr3a</i> transcripts in parental strains and progeny of test-cross P6497 X F ₁ (ACR10 X P7076).....	57
Figure 3.7 Relative expression profile of <i>Avr3a</i> gene among the hybrids obtained from P6497 X F ₁ -81 test-cross vs. virulence assay on soybean <i>Rps3a</i>	61
Figure 3.8 Analysis for allele-specific expression of <i>Avr3a</i> , illustrating the release of gene silencing of the P7076 allele in progeny from the test-cross P6497 X F ₁ (ACR10 X P7076).....	63
Figure 3.9 DNA marker analyses to determine hybrids from a cross of <i>P. sojae</i> strains P6497 X ACR10.....	66
Figure 3.10 Self-fertilized and hybrid progeny from the cross of <i>P. sojae</i> strains P6497 X ACR10.....	66
Figure 3.11 Photograph of virulence assay results of two different F ₁ hybrids of P6497 X ACR10 cross.....	67

Figure 3.12 <i>Avr3a</i> gene expressions of F ₁ hybrids of P6496 X ACR10 detected by RT-PCR.....	68
Figure 3.13 Mitochondrial DNA marker analyses of parental strains and F ₁ hybrids from a cross of <i>P. sojae</i> strains P6497 X ACR10.....	70
Figure 3.14 Representative virulence assays photographs of self-fertilized progeny.....	73
Figure 3.15 Parental <i>P. sojae</i> strains P6497 and ACR10 produce self-fertilized progeny that are true breeding with respect to the <i>Avr3a</i> virulence trait and <i>Avr3a</i> gene expression.....	74
Figure 3.16 Representative virulence assays of F ₂ progeny generated from self-fertilization of virulent and avirulent F ₁ , from a cross of <i>P. sojae</i> strains P6497 X ACR10.....	75
Figure 3.17 Analysis by RT-PCR of <i>Avr3a</i> gene expression among F ₂ progeny from a cross of <i>P. sojae</i> strains P6497 X ACR10.....	76

List of Abbreviations

A	Avirulent
aa	Amino Acid
ATP	Adenosine Triphosphate
<i>Avr</i>	Avirulence Gene
bp	Base Pair
BSA	Bovine Serum Albumin
CAP	Cleaved Amplified Polymorphism
cDNA	Complementary Deoxyribonucleic Acid
cv	Cultivar
dEER	Aspartate-Glutamate-Glutamate-Arginine
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetate
ETI	Effector Triggered Immunity
F ₁	First Generation
F ₂	Second Generation
gDNA	Genomic Deoxyribonucleic Acid
h	Hour(s)
Kb	Kilo Base
L	Litre
Liq N ₂	Liquid Nitrogen
LRR	Leucine Rich Repeat

M	Molar
MAPM	Microbe Associated Molecular Pattern
Mb	Mega Base
mg	Milligram
mL	Millilitre
mM	Milli Molar
MQ	Milli-Q
mRNA	Messenger Ribonucleic Acid
mt	Mitochondrial
NBS	Nucleotide Binding Sequence
°C	Degrees Celsius
PAMP	Pathogen Associated Molecular Pattern
PCR	Polymerase Chain Reaction
PRR	Pattern Recognition Receptor
PTI	PAMP Triggered Immunity
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
QTL	Quantitative Trait Loci
R-gene	Resistance Gene
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
Rps	Resistance Gene to <i>Phytophthora sojae</i>
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RXLR	Arginine-Any-Lysine-Arginine

SDS	Sodium Dodecyl Sulphate
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
V	Virulent
v/v	Volume/Volume
w/v	Weight/Volume
μg	Microgram
μL	Microliter

Chapter 1: Introduction

1.1. Biology of *Phytophthora sojae*

1.1.1. Evolutionary History

Associations occurring within the phyllosphere or rhizosphere between plants and microorganisms such as fungi, oomycetes, bacteria, viruses, and parasites have the potential to cause disease in plants. Among all these microbes, genera of oomycetes such as *Bremia*, *Peronospora*, *Phytophthora*, and *Pythium* cause highly destructive diseases in agriculturally important crops. Oomycetes are similar to fungi in morphology but the evolutionary history indicates that these organisms evolved independently. Oomycetes are classified in the kingdom Straminopila (Figure 1.1) (Qutob et al., 2009). These organisms are eukaryotes and are more closely related to brown algae and diatoms than to fungi (Kamoun, 2003). Although oomycetes look like true fungi in morphology, their motile zoospores have two flagella (Bikonts) whereas true fungi with zoospores have just a single flagellum (Unikonts). The two flagella on zoospores of oomycetes are unique ('tinsel' and 'whiplash'); therefore, oomycetes belong to Heterokonts, a special group of bikont organisms. Another difference between oomycetes and true fungi is the cell wall composition. The cell wall of fungi is made up of chitin but the major portion of the cell wall of oomycetes is made up of cellulose and polymers of beta-glucan. Also, unlike most fungi, *Phytophthora* are predominantly diploid, rather than haploid, for most of their life cycle (Tyler and Gijzen, 2014).

Phytophthora is one of the best-studied genera of oomycetes and species of this genus include the most notorious and devastating plant pathogens (Kamoun, 2003; Qutob et al., 2002). The first known outbreak of root rot disease of soybean occurred in North America in early 1950s and *P. sojae* was identified as a causative agent (Erwin and Ribeiro, 1996; Hildebrand, 1959; Kaufmann and Gerdemann, 1958). It remains uncertain whether *P. sojae* is native to Asia or North America, but there is evidence that supports the hypothesis that it originated in North America (MacGregor et al., 2002; Tyler and Gijzen, 2014). *P. sojae* is one of about 120 species of the genus *Phytophthora* (Tyler and Gijzen, 2014).

Classification of *P. sojae* Kaufm. & Gerd. [from (Tyler, 2007)]

Superkingdom- Eukaryota

Kingdom- Stramenopila

Phylum- Oomycota

Class- Peronosporomycetidae

Order- Pythiales

Family- Pythiaceae

Genus- *Phytophthora*

Species- *Phytophthora sojae*

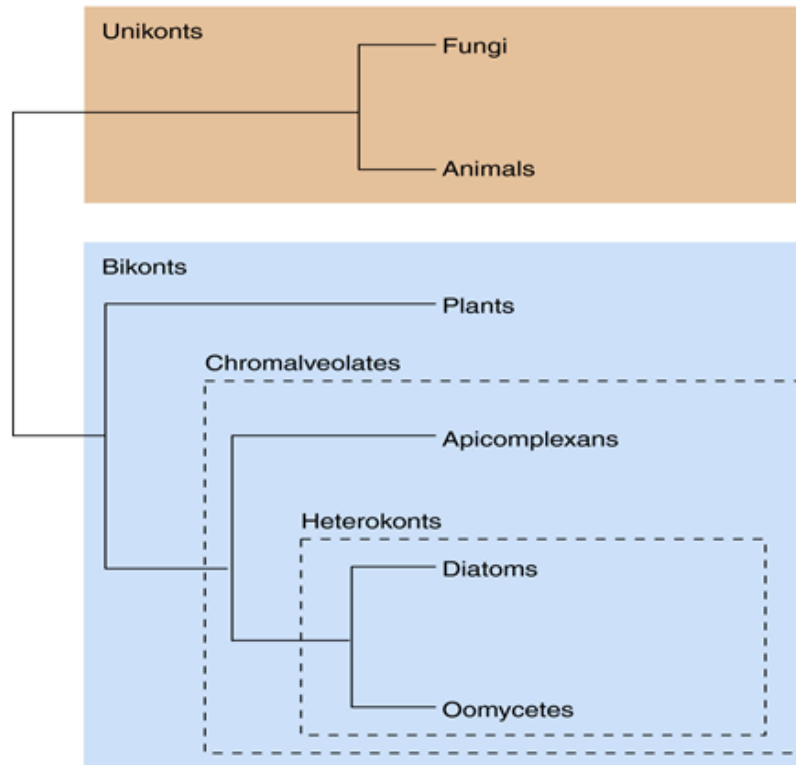


Figure 1.1 Eukaryotic phylogeny based on morphology (Gijzen, 2013, adapted from poster). Bikonts- two flagella present on motile spores; Unikonts- single flagellum on motile spores; heterokonts- possess unique ‘tinsel’ and ‘whiplash’ flagella on zoospores.



Figure 1.2 Colony morphology of *P. sojae*. *P. sojae* strain P6497 on V8 juice medium after 5 days incubation at 25°C.

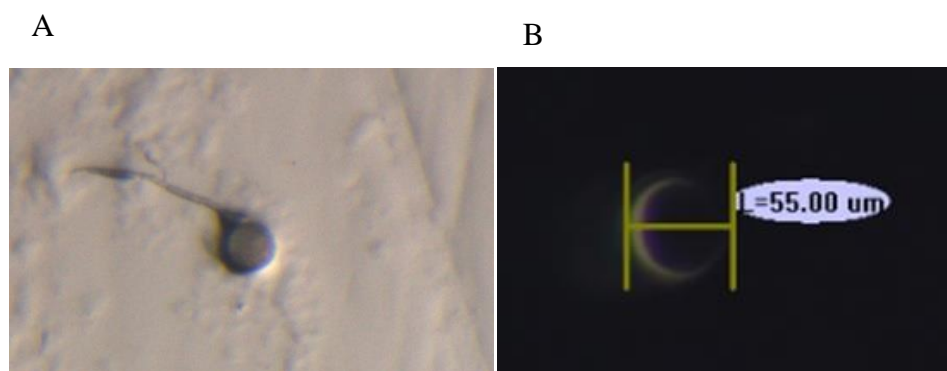


Figure 1.3 Oospore of *P. sojae*. (A) Germinating oospore under 60X magnification of stereomicroscope isolated by out-crossing of *P. sojae* strains P6497 X ACR10 on water agar plate. (B) Measurement of diameter of oospore using digital camera attached with stereomicroscope under 60X.

1.1.2. Life cycle of *P. sojae*

The life cycle of *P. sojae* is presented in the Figure 1.4. The diploid nature of oomycetes affects their evolution and host adaptation. The only haploid cells of *P. sojae* are the germ cells within the oogonium and antheridium. *Phytophthora sojae* produces three different types of spores including both sexual (oospores) and asexual (zoospores and chlamydosopores) spores. The union of antheridium (male gametophyte) with oogonium (female gametophyte) allows the nuclei from the antheridium to fertilize the egg in the oogonium and develop the oospore. Oospores can remain dormant in the soil for many years, even in adverse environmental conditions, causing persistence of the disease (Gijzen, 2009; Judelson, 2008; Tyler, 2007). Due to the homothallic nature of *P. sojae*, i.e. individual strains can produce both male and female gametes, and are self-fertile, oospores can develop by both self-fertilization of one strain or out-crossing of two different strains of *P. sojae* (Tyler and Gijzen, 2014).

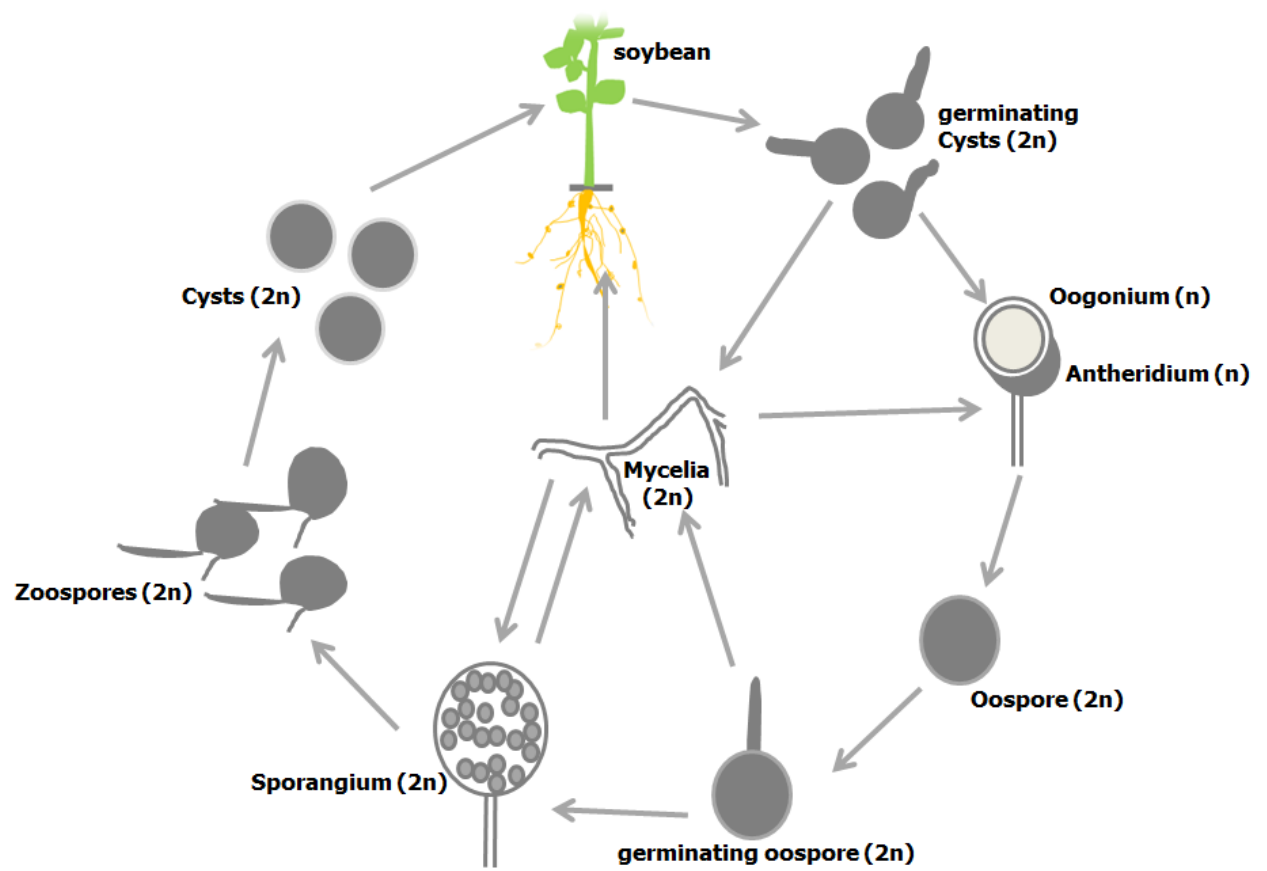
Zoospores are kidney shaped asexual spores and are the most infectious form of the pathogen under wet conditions. A mature sporangium can release 10-30 water motile zoospores. Zoospores do not have a cell wall and are short lived. Chlamydosopores develop in the old cultures and dead plant tissue (Tyler, 2007). Chemotactic factors in the roots of soybean plants attract zoospores to the root surface of soybean (Morris and Ward, 1992; Tyler, 2002; Tyler et al., 1996). Two isoflavones involved in this chemo attraction are daedzein and genestein (Morris and Ward, 1992). As soon as a zoospore reaches the root surface, it loses the flagella, adheres to the root surface, encysts, and develops a germ tube to infect the host (Gijzen and Qutob, 2009; Tyler, 2007). Zoospores

can also spread on and infect the plant phyllosphere (parts of the plant above the ground level). Germinating zoospores then develop a special structure called as appressorium at the point of penetration (Enkerli et al., 1997). After penetrating the epidermal layer, the hyphae spread intercellularly developing finger-like haustoria through the host epidermis and cortex. In the susceptible plants, the hyphae penetrate the intracellular cortex, endodermis, and then stele, where they grow to a massive proliferation and rapidly spread the infection (Gijzen and Qutob, 2009; Tyler, 2007). The pathogens produce toxins (necrosis inducing proteins) that kill host cells (Gijzen and Nurnberger, 2006). After cell necrosis, the host plants show the disease symptoms and start to wilt and die. The hyphae of *P. sojae* develop oogonia and antheridia, in which meiosis takes place to form haploid gametes, and after fusion of these sexual gametes, diploid sexual oospores are produced inside the host tissues. Oospores can lie dormant in the soil for many years. Oospores can germinate and develop directly into a sporangium to produce zoospores in favourable environmental conditions (warm and wet soil). The oospores can also germinate to produce hyphae, which can infect a new host.

1.1.3. Pathogenesis

Phytophthora sojae is a widespread soil borne hemibiotrophic plant pathogen that can live both as biotroph that can infect live plants and necrotroph which can kill and live in dead plant tissues (Erwin and Ribeiro, 1996; Gijzen and Qutob, 2009). It has a narrow host range including soybean and lupines (*Lupinus* spp.), but soybean is the only economically important host (Gijzen and Qutob, 2009; Tyler, 2007). Favourable environmental conditions, such as a warm environment (25°C-30°C), poor drainage,

flooding, soil compaction, no-till practices, wet conditions, and clay soils foster the disease to spread in the field. *Phytophthora sojae* can infect any part of the host plant at any developmental stage, but mostly seedlings and younger plants are affected (Dorrance and Niklaus, 2009; Schmitthenner, 1985; Schmitthenner, 2000). Usually *P. sojae* infects the plant starting from roots and the infection continues towards the upper parts (stem) but sometimes it can directly infect the aerial parts of the plant. Flooding and rain helps to spread the disease. During host infection, *P. sojae* progresses from a biotrophic to a necrotrophic growth mode. During the biotrophic phase, infected host cells remain alive, whereas necrotrophic growth results in host cell death. Most pathogen biomass is produced during necrotrophic growth, when the infection spreads rapidly. Infected plants in the field show symptoms of disease 1-2 weeks after infection. Water soaked lesions develop in infected tissues that causes brown coloured rot in the root and stem. The leaves of infected plants change colour to yellow, and eventually the whole plant becomes an orange-brown colour, then wilts and dies. The severity of the disease depends on the resistance capacity of the host plants (Gijzen and Qutob, 2009).



n: haploid, 2n: diploid

Figure 1.4 Life cycle of *P. sojae*.

1.2. Economy

Soybean is one of the major crops in Canada and around the world. An estimated >230 million tonnes per annum of soybeans are produced globally (Hartman et al., 2011). *Phytophthora sojae* causes widespread problems in soybean growing areas around the world but especially in North America. The disease causes around 10^9 kg of soybean losses in North America each year, which is equivalent to more than \$300 million loss per year (Schmitthenner, 2000; Wrather and Koenning, 2006). In the United States alone, *P. sojae* causes nearly \$200 million in annual yield losses (Lin et al., 2014). Worldwide, the disease causes approximately \$1-2 billion losses per annum (Gijzen and Qutob, 2009; Tyler, 2007). Canadian losses to this disease are estimated to be in the range of \$50 million a year (AAFC, Innovation Express, 2014).

1.3. Control of *P. sojae*

Tillage and tiling help to control soil drainage, spread and elimination of oospores, and reduce *P. sojae* infection on the field. Tiling is an agricultural practice to remove excess water from soil and tillage is the soil preparation process by using tools and some mechanical agitation for the plant cultivation (Schmitthenner, 2000; Workneh et al., 1999). Although oospores can survive for a long time, rotation of the crop in the field is one alternative for disease control. Another is to sow resistant cultivars in infested fields (Schmitthenner, 2000; Williams and Schmitthenner, 1962). Since *P. sojae* is not a fungus, many fungicides are an ineffective treatment. Another problem of treatment is that the pathogen inhabits the soil and infects the plant in root parts under the surface. Chemical treatment of seed (metalaxyl) and soil [CaCl_2 , $\text{Ca}(\text{NO}_3)_2$] helps to prevent the

disease but the costs are high. Variation of *P. sojae* also makes management difficult, because it can evolve rapidly into new strains that defeat previously resistant soybean cultivars (Chamnanpant et al., 2001; Gijzen and Qutob, 2009; Schmitthenner, 1985; Tyler and Gijzen, 2014). Partial resistance of soybean to *P. sojae* is controlled by quantitative trait loci (QTL), and helps to minimize the severity of the disease. Though partial resistance is believed to be more durable than *R*-gene mediated resistance, it is more difficult to select for in a breeding program (Gijzen and Qutob, 2009; Tyler and Gijzen, 2014). Therefore, *R*-gene (*Rps* gene) mediated resistance are widely used in commercial soybean production and are easier to manage in breeding programs than QTL (Slaminko et al., 2010).

1.4. Genomics of *P. sojae*

Phytophthora sojae is a model species of the genus *Phytophthora*. The whole genome sequencing was completed in 2004 and published in 2006 with a whole genome size of 95 Mb (Govers and Gijzen, 2006; Tyler and Gijzen, 2014; Tyler et al., 2006). Genetic analysis of *P. sojae* has advanced rapidly but scientists are still unsure about some important characteristics, such as the number of chromosomes; estimates of the total number of chromosomes is in the range from 12-15 (Gijzen and Qutob, 2009; Hansen et al., 1986). Genome sequencing resulted in 26,584 predicted genes (Tyler and Gijzen, 2014). The mitochondrial genome sequences were also determined during the whole genome sequencing and the mitochondrial genome size is 42,977 bp with 37 protein encoding genes (Martin et al., 2007).

1.4.1. Genetic crosses and inheritance of *P. sojae*

P. sojae is a homothallic organism. Self-fertilized oospores can develop from one strain, while out-crossing between strains results in F₁ hybrids. Crossing different strains has potential to produce a diverse group of progeny. Until recently, it was difficult or impossible to distinguish hybrid progeny from self-fertilized progeny. Researchers used molecular markers for this purpose and the first hybrids of *P. sojae* were reported in the 1980s (Layton and Kuhn, 1988). Later, many different DNA markers were developed to identify *P. sojae* hybrid progeny. To study the segregation pattern of genetic loci and construct a genetic map of linkage groups, outcrosses can be carried out between two parental strains and hybrids can be determined by using molecular markers (May et al., 2002; Tyler et al., 1995; Whisson et al., 1995). Many different traits and genetic markers have been tracked in the F₁ and F₂ progeny. The segregation patterns usually follow Mendelian rules but exceptions do occur (MacGregor et al., 2002; Qutob et al., 2013; Qutob et al., 2009). Parasexual mechanisms such as mitotic gene conversion may cause loss of heterozygosity in F₁ or F₂ progeny, and result in unusual inheritance patterns (Chamnanpant et al., 2001; Förster and Coffey, 1990; Francis et al., 1994; Tyler and Gijzen, 2014).

1.4.2. Gene-for-gene interactions between *P. sojae* *Avr* genes and soybean *Rps* genes

All plant cells have innate immunity to defend against different pathogenic microorganisms. Plants develop diverse sets of defenses including physical and chemical barriers, antimicrobial enzymes, antimicrobial chemicals (phytoalexins, reactive oxygen species, and phytoanticipins), and may undergo programmed cell death and activate

signalling systems at the site of infection (Ausubel, 2005; Jiang and Tyler, 2012; Jones and Dangl, 2006). The process of innate immunity involves extracellular receptor molecules called pathogen or pattern recognition receptors (PRRs) that respond to conserved or slowly evolving microbial- or pathogen-associated molecular patterns (MAMPS or PAMPs), such as lipopolysaccharide, flagellin, or chitin, to trigger immunity (Ausubel, 2005; Boller and Felix, 2009). Plant immunity triggered by PAMPs is called PAMP- or pattern-triggered immunity (PTI) (Tsuda and Katagiri, 2010). In addition to extracellular receptors plant cells contain cytoplasmic resistance (R) proteins with C-terminal leucine rich repeats (LRR), central nucleotide-binding site (NBS) domains and N-terminal domains which are not conserved (Sandhu et al., 2004). Soybean *R* genes are believed to encode immune receptor proteins that reside in the cytoplasm and that are specific to pathogen effectors that enter the plant cell. Soybean *R* genes specific to *P. sojae* are called *Rps* genes and determine strain specific resistance.

Phytophthora sojae secretes effector molecules that enhance infection by suppressing host defense mechanisms. However, particular effector molecules named avirulence (*Avr*) factors may be sensed by plant resistance (R) proteins and cause effector triggered immunity (ETI) (Jones and Dangl, 2006; Qutob et al., 2013). There are 14 mapped *Rps* genes from soybean (Sandhu et al., 2004). Although *Rps* genes are extremely effective in providing immunity against infection, the *Rps* gene-mediated immunity is not durable due to gain of virulence among pathogen strains through evolution (Kasuga and Gijzen, 2013). The effectors may act on apoplast, plasma membrane or cytoplasm of plant cells (Jiang and Tyler, 2012). PTI is responsive to extracellular effectors and molecules whereas ETI responds to intracellular effector

molecules (Jiang and Tyler, 2012). Disease outcome of *P. sojae* infection on soybean depends on the gene-for-gene interaction of pathogen avirulence (*Avr*) genes and host resistance (*R*) genes (Shan et al., 2004). Both defense mechanisms (PTI, ETI) of plant cells prevent pathogen infection, suppress multiplication, and kill pathogens that have already entered the plant tissues.

1.4.3. *Avr* genes of *P. sojae*

The advanced genetic and bioinformatic approaches and a high-quality reference genome assembly of *P. sojae* has helped in the identification of *Avr* genes (Jiang and Tyler, 2012; Tyler and Gijzen, 2014; Tyler, 2007). Sequence characteristics, polymorphism in the sequences, and association with virulence trait are the basis to select candidate *Avr* genes (Na et al., 2014). Moreover, outcrossing parental strains and tracking segregation in F₂ populations is an important map-based tool in identification of *Avr* genes of *P. sojae* (Gijzen et al., 1996; MacGregor et al., 2002; May and Ristaino, 2004; Tyler et al., 1995; Whisson et al., 1994; Whisson et al., 1995). All of the *P. sojae* *Avr* genes are predicted to encode secreted proteins with RXLR (Arg-any amino acid-Leu-Arg) and dEER (Asp-Glu-Glu-Arg) motifs. Other than RXLR and dEER motifs, the effector domain also contains additional conserved motifs in C-terminus which are W, Y, L, and K motifs with their own importance in function, adaptation and infection to host cells (Dou et al., 2008; Jiang et al., 2008). The W and F motifs are highly conserved tryptophan and tyrosine residues that form the hydrophobic core of the three helices fold, known as the WF fold. The K motif is a positively charged lysine residue (Jiang and Tyler, 2012). The *P. sojae* reference genome contains 453 predicted RXLR effectors,

which are called Avirulence homolog (Avh) proteins (Jiang et al., 2008; Qutob et al., 2009; Win et al., 2007). Known *P. sojae* Avr genes thus far include *Avr1a* (Qutob et al., 2009), *Avr1b* (Shan et al., 2004), *Avr1c* (Na et al., 2014), *Avr1d* (Na et al., 2013), *Avr1k* (Song et al., 2013), *Avr3a/5* (Dong et al., 2011b), *Avr3b* (Dong et al., 2011a), *Avr3c* (Dong et al., 2009), and *Avr4/6* (Dou et al., 2010).

The RXLR effector proteins are believed to suppress host defenses and promote disease (Qutob et al., 2009). The mechanism of entry of effector proteins to the plant cell cytoplasm is unknown but RXLR and dEER motifs downstream from the signal peptide are thought to play a role in host entry (Dou et al., 2008). Although the mechanism of entry is unknown, some research indicates that RXLR motif binds to phosphatidylinositol-3-phosphate [PtdIns (3)P] to enter the host cell (Kale et al., 2010), but other findings indicate that C-terminal domain has the lipid binding affinity rather than RXLR itself (Sun et al., 2013; Wawra et al., 2012; Yaeno et al., 2011). Recent results show that one Avr factor (*Avr1d*) binds to phosphatidylinositol-4-phosphate [PtdIns(4)P] rather than [PtdIns (3)P] and lipid binding activity was disrupted when C-terminal region was mutated but not the RXLR motif (Na et al., 2013).

The *Avr3a* gene of *P. sojae* was identified through genetic mapping and transcriptional profiling (Qutob et al., 2009). Genetic out-crossing of *P. sojae* strain P6497 (avirulent towards soybean *Rps3a* gene) and strain P7064 (virulent towards soybean *Rps3a* gene) and analysis of F₁ and F₂ progeny demonstrated that the avirulence segregated as a dominant Mendelian trait. Virulence is defined as the degree of damage by a pathogen to the host which is negatively correlated with host fitness (Sacristan and

Garcia-Arenal, 2008). Therefore virulent pathogens have ability to cause disease and avirulent pathogens do not have the ability to cause disease. Transcripts of *Avr3a* mRNA were detected in avirulent strains and progeny but not in the virulent strains and progeny (Qutob et al., 2009). The *Avr3a* gene of *P. sojae* encodes a predicted protein of 111 amino acids consisting of N-terminal signal peptide, a host targeting motif (RXLR), and a carboxy terminal effector domain. The *Avr3a* gene resides on a 10.8 kb DNA segment that displays copy number variation among strains of *P. sojae* (Figure 1.5). The sequence of the *Avr3a* allele shows variation among *P. sojae* strains (Figure 1.6) (Qutob et al., 2013; Qutob et al., 2009). *Phytophthora sojae* strains possessing *Avr3a* mRNA transcripts are avirulent on *Rps3a* plants but strains lacking *Avr3a* mRNA transcripts are virulent to soybean plants carrying *Rps3a* (Qutob et al., 2013).

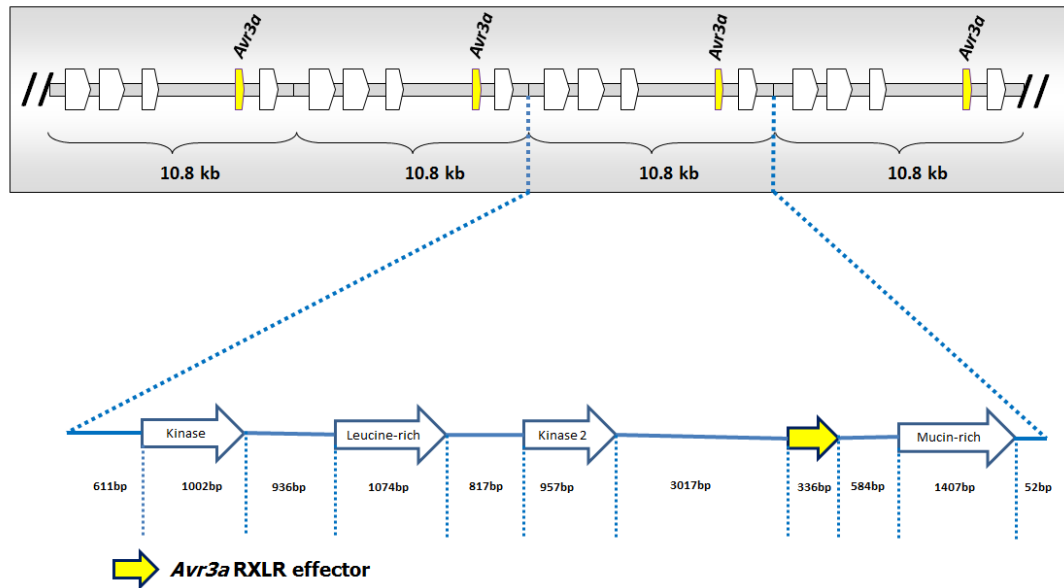


Figure 1.5 *Avr3a* gene model of *P. sojae* strain P6497. The *Avr3a* gene resides on 10.8 kb DNA segment and displays copy number variation among different strains. Yellow colour denotes *Avr3a* gene. Besides the *Avr3a* gene, the segment contains four other predicted genes, as illustrated (Qutob et al., 2009).

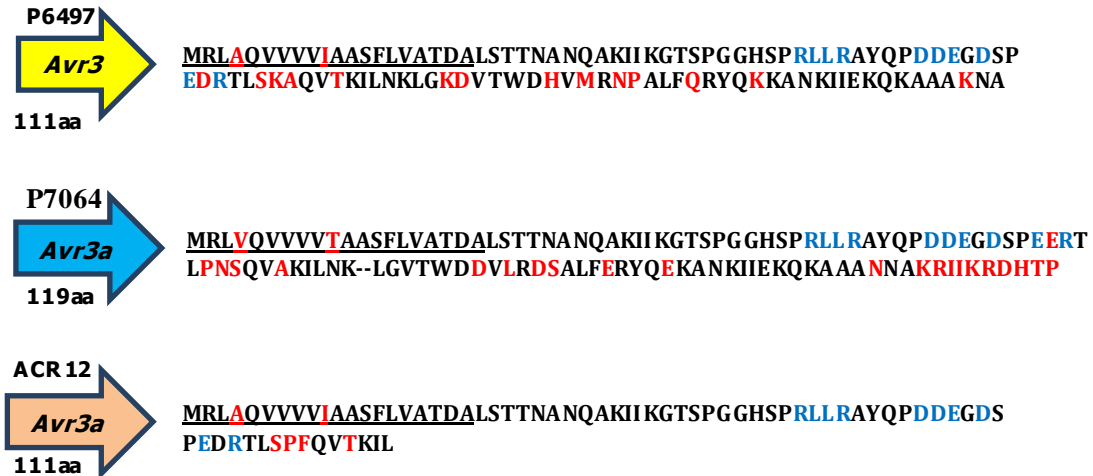


Figure 1.6 Predicted protein sequences of three alleles of the *Avr3a* gene identified among *P. sojae*. Strains 48FPA18, P6497, 25MEX4, ACR8, ACR9, ACR 10, ACR11, ACR 16, and ACR25 contain *Avr3a*-1; strains P7064, P7064, P7074, ACR21, and ACR24 contain *Avr3a*-2; and strains ACR12, P7076, and ACR20 contain *Avr3a*-3. The underlined amino acid sequences are signal peptide. Red colour indicates polymorphic amino acid residues. The RXLR-dEER motif is shown in blue (Jiang et al., 2008; Qutob et al., 2009).

Table 1.1 The *Avr3a* alleles in different strains of *P. sojae*.

<i>Avr3a</i> -allele type	<i>Avr3a</i> -1	<i>Avr3a</i> -1'	<i>Avr3a</i> -2	<i>Avr3a</i> -3
Copy number of <i>Avr3a</i> gene	4	4	1	1
<i>Avr3a</i> mRNA transcript	+	-	+	-
Virulence on <i>Rps3a</i>	A	V	A	V
<i>P. sojae</i> strains	48FPA18, P6497, 25MEX4, ACR8, ACR9, ACR11, ACR25	ACR10, ACR16	ACR12, P7076, ACR20	P7064, P7074, ACR21, ACR24

⁺detectable mRNA transcript, ⁻no detectable mRNA transcript, ^Aavirulent, ^Vvirulent

(Qutob et al., 2009).

1.5. Gain of virulence changes to *P. sojae* Avr genes

Pathogens go through continuous evolutionary changes and at the same time, host plants co-evolve to defend themselves against disease. Many species of filamentous fungi and oomycetes evolved to colonize and grow within plant tissues. Some of these organisms can have beneficial effects on plants but others cause diseases in wild plants and agricultural crops (Raffaele and Kamoun, 2012). To escape from *R*-gene mediated host immunity, oomycetes and filamentous fungi acquire virulence by adaptive changes to their *Avr* genes (Jiang and Tyler, 2012; Vleeshouwers and Oliver, 2014).

Recent results show that gain of virulence changes to *P. sojae* *Avr* genes include epigenetic variation of gene expression (Kasuga and Gijzen, 2013). It has also been found that genetic outcrosses between particular strains of *P. sojae*, which differ in virulence on soybean plants with *Rps3a* results in transgenerational gene silencing and gain of virulence in progeny (Qutob et al., 2013). This could be due to epigenetic mechanisms that cause heritable changes in gene expression (phenotype) without changes in the DNA sequence (Goldberg et al., 2007). Epigenetic factors could cause the transgenerational inheritance and non-Mendelian segregations. Self-propagating factors (Tyler and Gijzen, 2014) such as DNA methylations, histone modifications, and small RNA molecules could account for epigenetic inheritance (Gavery and Roberts, 2010). Epigenetic traits can also propagate through mitotic divisions, but for generational inheritance, propagation through meiosis is essential. Research in epigenetic phenomena has gained momentum as more examples of unusual inheritance patterns have emerged (Richards, 2006). Other processes or effects that may be under epigenetic control include the activity of transposons,

position-effect variegation, X-chromosome inactivation, paramutation, parental imprinting, and transgene silencing (Richards, 2006). Epigenetic mechanisms play roles in phenotypic plasticity of organisms, soft inheritance, and response to environmental stressors (Allendorf et al., 2010; Bastow et al., 2004; Richards et al., 2010; Richards, 2006; Schrey et al., 2012).

An epigenetic transfer of information from one allele of a gene to another that causes a heritable change is called paramutation. In paramutation, heritable changes transfer meiotically to subsequent generations and are maintained stably (Della Vedova and Cone, 2004). It has been described as a breakdown of Mendel's first law (Brink, 1973). A newly silenced allele called a paramutagenic allele continues to silence other naive alleles (paramutable) in the subsequent generations (Chandler and Alleman, 2008; Pilu, 2011). Some alleles which are not silenced by paramutagenic allele are known as neutral alleles (Della Vedova and Cone, 2004). DNA methylation, change in chromatin structure, and noncoding RNA are found to be associated with the establishment and maintenance of paramutation. Furthermore, paramutation is also affected by mutation in different genes (Dorweiler et al., 2000; Stam, 2009). In the past, paramutation was defined as "conversion-type inheritance" (Coe, 1966), but later gene conversion is defined as the nonreciprocal recombination process within a locus during meiosis and is unrelated to paramutation (Jinks-Robertson and Petes, 1985). It has been described that the infrequent known occurrences of paramutation was due to the limited diversity of experimental organisms used for genetic studies (Brink, 1956).

1.6. Thesis Hypotheses

Recent results demonstrated transgenerational gene silencing of *Avr3a* and the gain of virulence on soybean plants carrying *Rps3a* gene. To continue this research, I hypothesize that:

- I. Transgenerational gene silencing of *Avr3a* is meiotically stable over multiple generations.
- II. Gene silenced alleles of *Avr3a* gain the capability to silence expressed alleles of other strains. Specifically, the silenced *Avr3a* allele of strain P7076 is paramutagenic and is expected to silence the expressed *Avr3a* allele of *P. sojae* strain P6497 as a paramutale allele following a test-cross between P6497 X F₁ (P7076 X ACR10) hybrid. Likewise, the silenced *Avr3a* allele of ACR10 will silence expressed *Avr3a* allele of P6497.
- III. As the *Avr3a*^{P7076} allele was silenced by the *Avr3a*^{ACR10} allele, the *P. sojae* strain P6497 *Avr3a* allele is also subject to gene silencing when crossed with strain ACR10.

1.7. Thesis Objectives

To address each of the above mentioned hypotheses, the objectives of present research are:

- I. To develop further (F₄ and F₅) generations of ACR10 X P7076 by performing self-fertilization. The F₃ cultures will be self-fertilized to develop F₄ progeny and similarly F₅ progeny will be developed from F₄ cultures. The *Avr3a* gene

expression will be analyzed by RT-PCR and virulence will be tested on soybean plants carrying *Rps3a* gene.

- II. To perform a test cross of F₁ (ACR10 X P7076) X P6497 and determine the *Avr3a* expression and virulence of the hybrid progeny. *P. sojae* is a homothallic organism and produces both hybrid and self-fertilized progeny. Therefore, hybrid progeny containing the genotype of (*Avr3a*^{P6497}/*Avr3a*^{P7076}) and (*Avr3a*^{P6497}/*Avr3a*^{ACR10}) will be identified using DNA markers.
- III. To perform a cross of *P. sojae* strains P6497 X ACR10 and determine the segregation of *Avr3a* gene expression and virulence in the progeny.

Chapter 2: Materials and Methods

2.1 *Phytophthora sojae* strains

Cultures of *P. sojae* parental strains (P6497, ACR10, P7076), F₁ (F₁-62, F₁-81) and F₃ (F₃-40, F₃-60, F₃-83, F₃-87) progeny from the cross ACR10 X P7076, were available in Dr. Gijzen's laboratory, Agriculture and Agri-Food Canada. The origin of the parental strains used in this study is shown in Table 2.1. For short term (up to one year) storage, cultures were maintained on 2.5% (v/v) vegetable juice (V8) agar medium at 16°C in the dark. For prolonged storage, parental strains and hybrid progeny were cryo-preserved in liquid nitrogen.

2.2 Plant materials

Soybean (*Glycine max*) cultivar Williams (*rps*) and the corresponding isoline L83-570 (*Rps3a*) were grown in Southern Crop Protection and Food Research Centre (SCPFRC), Agriculture and Agri-Food Canada. The seeds grown in SCPFRC were used for virulence assays of parental strains and offspring of *P. sojae*.

2.3 Culture and cross of *P. sojae*

Phytophthora sojae strains were cultured by transferring 5 mm diameter mycelial plugs cut from the growing edge of a culture onto 26% (v/v) vegetable juice (V8) agar (regular) medium and incubated at 25°C for 7 days in the dark. Crossing media plates [2.5% (v/v) V8 juice agar] supplemented with β -sistosterol (10 µg/mL), kanamycin (50 µg/mL), ampicillin (100 µg/mL), and rifampicin (10 µg/mL) were freshly prepared. To

cross two different strains or to perform self-fertilization of *P. sojae*, fresh culture from growing edge of regular V8 juice medium (5 mm square) was transferred aseptically on the centre of the crossing plate and incubated at 25 °C for 7 days in the dark. After incubation, cultures (different strains for out-crossing, and same strain for selfing) grown on separate plates were homogenized together and co-cultivated on crossing medium plate at 25 °C in the dark for 5 weeks. Cultures were homogenized using a 10 mL sterile syringe with 18-gauge needle. Darkness was maintained using aluminium foil to wrap the plates.

2.3.1 Generation of F₄ and F₅ progeny from *P. sojae* cross ACR10 X P7076

Selected F₃ progeny with the genotype $Avr3a^{P7076}/Avr3a^{P7076}$ were available from the cross ACR10 X P7076. These cultures were self-fertilized to produce F₄ and F₅ progeny. The original ACR10 X P7076 cross was previously performed and the progeny were maintained in the laboratory (Qutob et al., 2013). To perform studies in this thesis, a total of four F₃ individuals (F₃-40, F₃-60, F₃-83, and F₃-87) were revived from cryo-storage, and self-fertilized to generate F₄ progeny; similarly, F₅ progeny were developed from the F₄ progeny (Figure 2.2).

Table 2.1 Strains of *P. sojae* and their original sites

Strain (Race)	Site of origin	Investigator	Virulence on Soybean <i>Rps3a</i> gene
P6497 (R2)	Stoneville, MS, USA	B. Keeling	Avirulent
ACR10 (R10)	Stoneville, MS, USA	B. Keeling	Virulent
P7076 (R19)	Stoneville, MS, USA	B. Keeling	Avirulent

(Dong et al., 2011b)

Table 2.2 Culture media used for *P. sojae*

Culture media	Ingredients (600 mL)	Purpose of media
26% (v/v) V8 juice agar	V8 juice: 156 mL, Agar: 8.4 g, CaCO ₃ : 1.6g, MQ-water: 440 mL	Routine growth and maintenance
2.5% (v/v) V8 juice agar	V8 juice: 15 mL, Agar: 8.4 g, MQ-water: 585 mL (for crossing, 10 µg/mL β- sistosterol is added)	Short term storage crossing of <i>P. sojae</i>
0.9% (w/v) V8 juice agar	V8 juice: 156 mL, Agar: 5.4 g CaCO ₃ : 1.6 g, MQ-water: 440 mL	Virulence assay
1.5% (w/v) water agar	Agar: 9 g, MQ-water: 440 mL β-sistosterol: 0.006 g (10 µg/mL)	Oospores isolation (10 µg/mL rifampicin is added after autoclaving)

(Ribeiro, 1978)

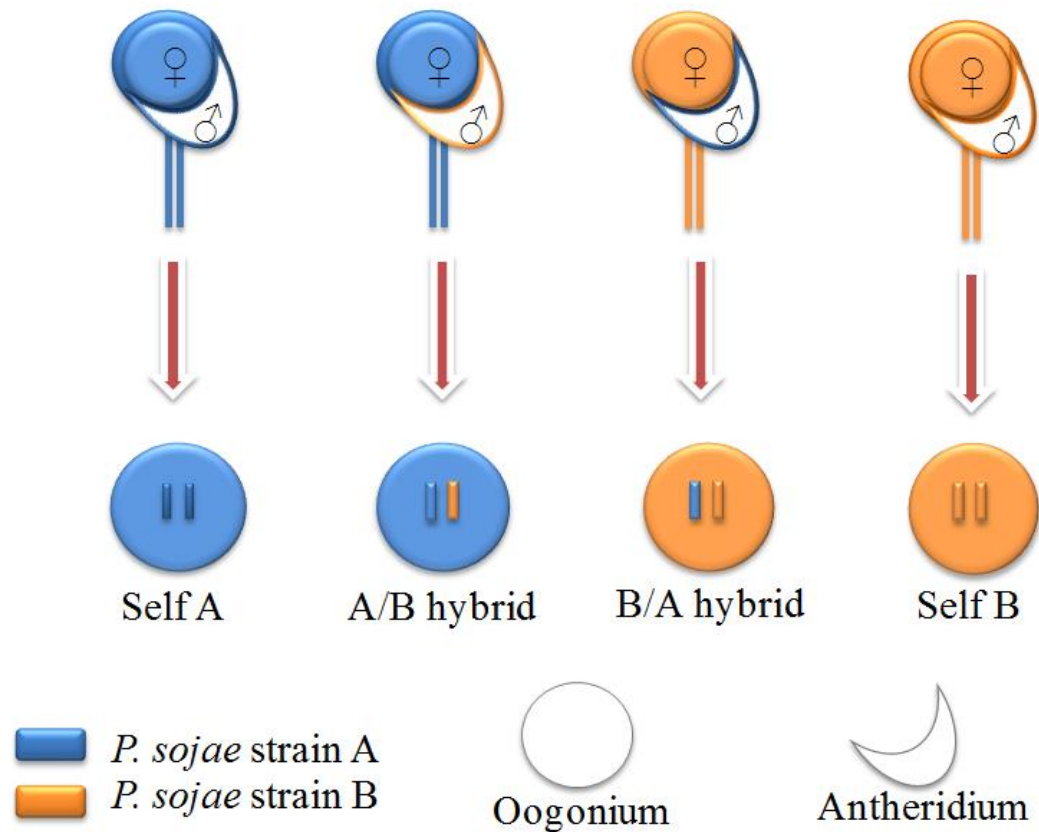


Figure 2.1 *P. sojae* is a homothallic diploid organism. Blue and orange colours denote two different strains of *P. sojae*. From the out-crossing of two different strains (A and B), four possible offspring are generated by fertilization of oogonium (♀) and antheridium (♂). Selfing of two similar strains results in homozygous offspring and out-crossing of two different strains results in heterozygous offspring.

2.3.2 Test-crossing of *P. sojae* F₁ (ACR10 X P7076) X P6497 (reference strain).

To study the inheritance of gene silencing of the *Avr3a* locus, an F₁ hybrid (*Avr3a*^{ACR10}/*Avr3*^{P7076}) from the cross ACR10 X P7076 was crossed with the reference strain P6497. Two biological replicates, P6497 X [ACR10 X P7076 (F₁-62)] and P6497 X [ACR10 X P7076 (F₁-81)] were performed.

2.3.3 Crossing of *P. sojae* strains P6497 X ACR10

Phytophthora sojae strains P6497 and ACR10 are sequence identical at the *Avr3a* locus but differ in expression of *Avr3a* and in virulence towards soybean carrying *Rps3a* gene. To study the *Avr3a* gene expression and inheritance of virulence in hybrids of these two strains a cross of P6497 X ACR10 was performed.

2.4 Isolation of oospores

All the steps in isolation of oospores were performed aseptically at a laminar flow bench. Out-crossing or self-fertilization was performed as mentioned above (section 2.3). After maturation, oospores were isolated by maceration, filtration, centrifugation, and other purification steps, described below (Gijzen and Qutob, 2009). After incubation for 5 weeks, four plates (for each crossing) of matured cultures were sliced using a sterile blade. Using a pre-chilled Waring commercial blender, the diced material from all four plates was blended together for 2 minutes (blending was stopped after one minute to allow the blender to cool to room temperature and before continuing another minute) with 100 mL of 4 °C sterile water.

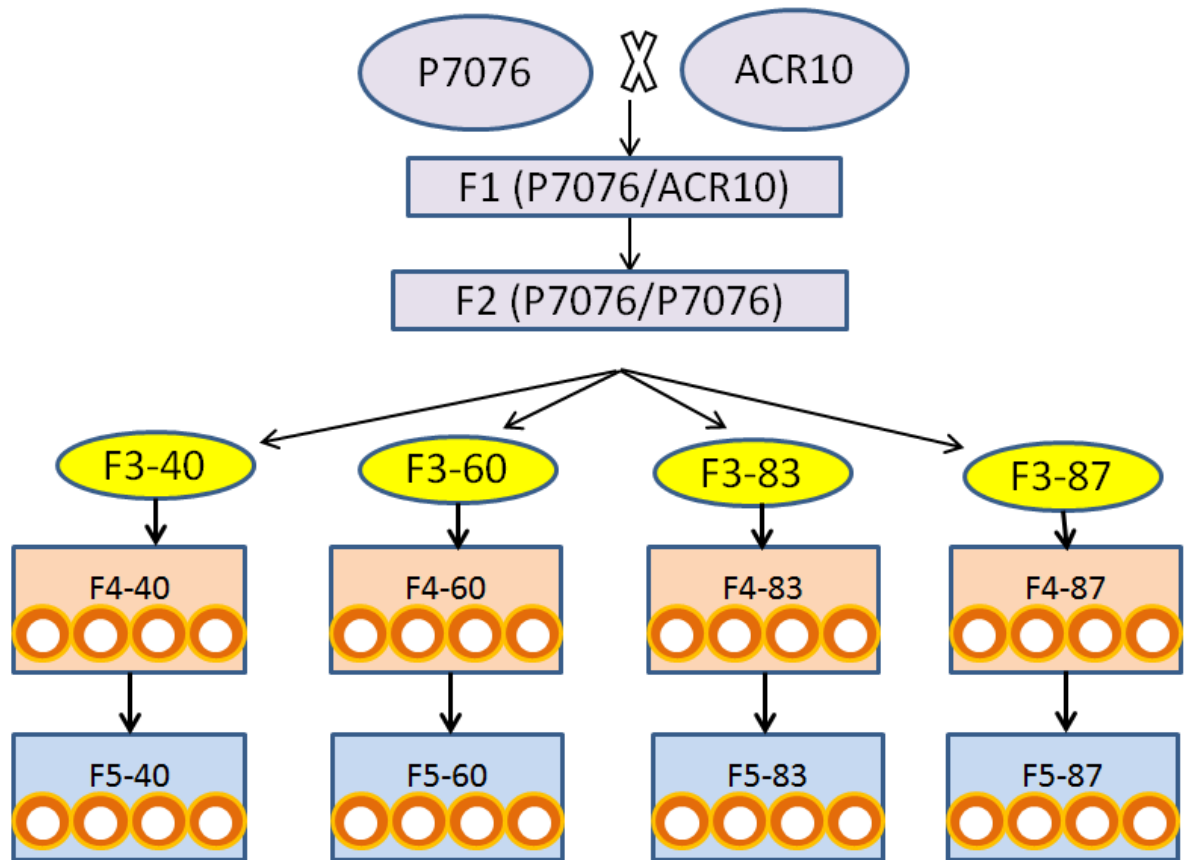


Figure 2.2 Schematics of Selfing. *P. sojae* cross ACR10 X P7076 was performed to develop F₁ progeny ($Avr3a^{ACR10}/Avr3a^{P7076}$). Subsequent F₂ progeny were developed by self-fertilization of F₁. By selecting F₂ progeny that were homozygous $Avr3a^{P7076}/Avr3a^{P7076}$, F₃ progeny of this genotype were developed. Four of the F₃ progeny were used to develop F₄ and F₅ progeny.

The cultures were then sieved through three sterile 75 µm nylon membrane to remove agar and mycelium. The filtrate was collected into a 50 mL sterile conical tube and frozen at -20 °C for 24 hours to kill the hyphae. In the following day, the culture was thawed at 45 °C water bath for 10 minutes and re-filtered using three sterile 75 µm nylon membranes. The filtrate was collected into a 50 mL sterile conical tube and centrifuged at 3000 rpm for 10 minutes. Mycelial fragments and agar which floated on the supernatant were removed using a sterile Pasteur pipette. A darkly coloured pellet of oospores can be observed after centrifugation. This pellet was then washed three times with sterile water.

The pellet containing oospores was treated with β -glucuronidase (2000 units/mL suspension) and incubated at 37 °C for 16 hours. After incubation, the oospore suspension was centrifuged and washed three times and re-suspended into 20 mL of sterile distilled water. Kanamycin (50 µg/mL) and ampicillin (100 µg/mL) were added to the suspension and oospores were spread on 1.5 % (w/v) water agar plates supplemented with 10 µg/mL β -sistosterol and rifampicin (10 µg/mL), approximately 500 oospores per plate (90 X 16 mm). The oospores on the water agar plates were incubated at 25 °C in the dark. Oospores begin to germinate after 2-4 days. Plates were checked every other day using a stereomicroscope (60X magnification). Germinating oospores were transferred from the water agar plate to regular V8 juice media using a sterile diamond-head transfer needle and incubated at 25 °C for 7 days in the dark. The cultures grown from pure isolated oospores were then used for further DNA/RNA work. A detailed flow chart of crossing of *P. sojae* strains and oospore isolation procedure is presented in the Figure 2.3.

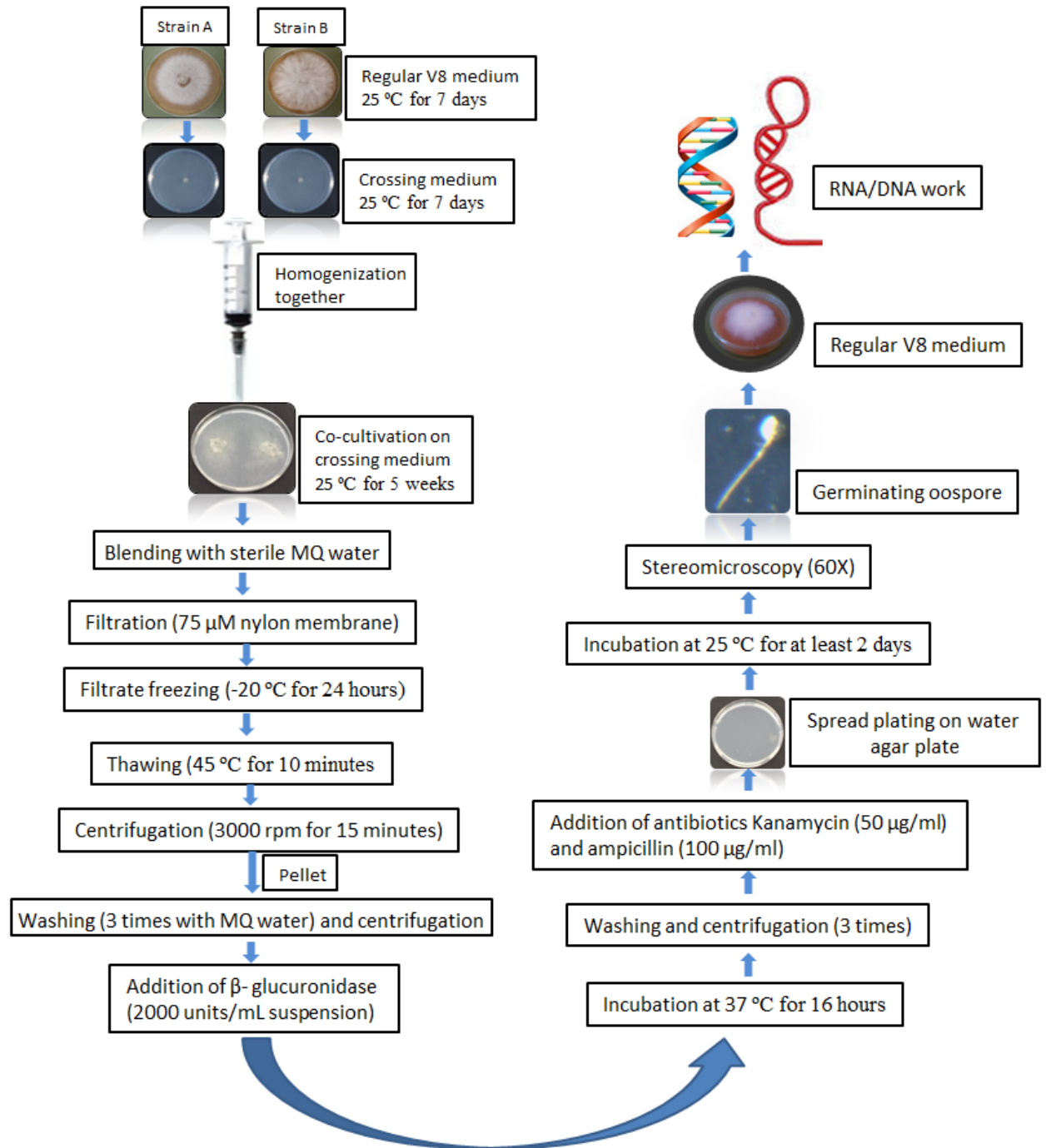


Figure 2.3 Diagrammatic flow chart of crossing of *P. sojae* and isolation of oospores

2.5 Genomic DNA isolation

Genomic DNA was extracted from mycelial cultures of *P. sojae* strains using phenol chloroform extraction followed by isopropanol precipitation (Sambrook and Russell, 2001). Cultures of *P. sojae* arising from single oospores were transferred to regular V8 medium and incubated at 25 °C for 7 days. A lawn of *P. sojae* mycelia growing on the surface of the V8 juice medium was collected by scraping with a sterile pipette tip (blunt end). The mycelia was then transferred into a 2 mL Eppendorf tube containing 1 mL mycelial extraction buffer and frozen at -20 °C for 24 hours. The mycelia extraction buffer contained: 200 mM Tris HCl (pH 8.5), 250 mM NaCl, 25 mM EDTA, 2% SDS, and sterile water. The next day, the frozen sample was thawed at room temperature. Phenol (750 µL) was added, and the sample was vortexed and centrifuged (13000 rpm for 15 minutes). The supernatant containing the DNA was collected and re-extracted again with phenol. The aqueous suspension was collected, and phenol: chloroform (1:1) was added, then the sample was centrifuged at 13000 rpm for 15 minutes. A solution (500 µL) of chloroform: isoamyl alcohol (24:1) was; added to the supernatant, and the sample was centrifuged at 13000 rpm for 15 minutes. The supernatant was collected and RNA was removed by adding RNase A (1.5 µL of 10 mg/ml). Isopropanol (60% volume of total DNA suspension) was added to the DNA suspension and the sample was kept at -20 °C overnight to precipitate the DNA. The DNA pellet was obtained by centrifugation (13000 rpm for 30 minutes at 4 °C). The DNA pellet was then washed with 70% (v/v) ethanol, centrifuged at 13000 rpm for 5 minutes, and air dried (10 minutes). The dried pellet was dissolved in a solution of 10 mM Tris HCl and 0.1 mM EDTA, heated at 65 °C for 10 minutes and stored at -20 °C.

2.6 Polymerase chain reaction (PCR)

For all PCR amplifications, a three step PCR method was applied. Initial denaturation temperature of the PCR reaction was 94 °C for 2 minutes. The three step cycle was: denaturation (94 °C for 40 seconds), annealing (annealing temperature was according to primers used for 40 seconds), and extension (72 °C for 1 minutes). After completion of all cycles, a 72 °C extension temperature was applied for 10 minutes. The number of PCR cycles varied according to the purpose of the experiment; for restriction digestion, 40 cycles were used, for RT-PCR, 30 cycles were used. The PCR amplification was performed using 15 ng genomic DNA as template. The PCR mixture (25 µL) was prepared using following reaction components: 0.2 mM dNTPs, 1.5 mM MgCl₂, 0.5 µM of each forward and reverse primers, 1.25 U Taq DNA polymerase and 1X PCR buffer as recommended by the supplier (Invitrogen, Life Technologies). Gel electrophoresis was performed to resolve products, which were visualized using a fluorescent dye (SYBR safe stain, Invitrogen).

2.7 Hybrid determination

To determine the hybrid progeny (P6497/ACR10 or P6497/P7076) from crosses of P6497 X F₁ (ACR10 X P7076), two different co-dominant cleaved amplified polymorphisms (CAPs) markers were analysed. The *Scaf-29-M2* sequence (448 bp) with strain-specific sequence polymorphisms was amplified using forward primer (5'-CCCTCGAGAACGCCAACTT-3') and reverse primer (5'-CCTCGCTCGCCTTCATCC-3'). The *Scaf-29-M2* sequences of strain ACR10 and P7076 possess a restriction site for the enzyme *EcoRV* but strain P6497 does not. Similarly, *Avh320* sequence (400 bp) was

amplified using forward primer (5'-AACGCTCTCGAAAGTGGC-3') and reverse primer (5'-AAAGAACTTCGACAG CC-3'). The *Avh320* sequences of strains P6497 and ACR10 have a restriction site for *ClaI* but strain P7076 does not. To track segregation of the *Avr3a*^{P6497} and *Avr3a*^{P7076} or *Avr3a*^{ACR10} alleles, forward primer (5'-GCTGCTTCCTTCCTGGTTGC-3') and reverse primer (5'-GCTGCTGCCTTTTGCTTCTC-3') were used and amplified *Avr3a* sequences were digested with restriction enzyme *AluI*. The *Avr3a* sequences of ACR10 and P6497 include a restriction site for *AluI* but the *Avr3a* sequence of P7076 does not (Figure 2.4).

2.8 Restriction Digestion

To analyse the CAPs markers, PCR amplification was performed as described above using 15 ng genomic DNA as template. For restriction digestion of amplified sequences, 10 µL volumes of amplified products and 10 µL enzyme mixtures (3 U of respective enzyme, buffer and BSA from New England Bio labs) were mixed and incubated according to the optimum temperature of the restriction enzymes. Gel electrophoresis was performed to resolve products, which were visualized using SYBR safe stain.







































(Genotype)	<i>Avh-320: ClaI</i>	<i>Scaf-29-M2: EcoRV</i>	<i>Avr3a: AluI</i>
P6497	268 bp  132 bp 	 448 bp	 163 bp  130 bp
ACR10	268 bp  132 bp 	 279 bp  169 bp	 163 bp  130 bp
P7076	400 bp 	 279 bp  169 bp	 293 bp
ACR10/P7076	400 bp  268 bp  132 bp 	 279 bp  169 bp	 163 bp  130 bp
P6497/ACR10	268 bp  132 bp 	 448 bp  279 bp  169 bp	 163 bp  130 bp
P6497/P7076	400 bp  268 bp  132 bp 	 448 bp  279 bp  169 bp	 293 bp  163 bp  130 bp

Figure 2.4 Restriction patterns of DNA markers used. The hybrids

$Avr3a^{P6497}/Avr3a^{ACR10}$ and $Avr3a^{P6497}/Avr3a^{P7076}$ after test-cross of P6497 X [F₁ (ACR10

X P7076)] were identified using three CAPs markers and three restriction enzymes.

Coloured bands represent DNA fragments of different size after restriction digestion.

2.9 Extraction of RNA and reverse transcriptase polymerase chain reaction (RT-PCR)

To study the expression of the *Avr3a* gene of *P. sojae* strains, total RNA was extracted from mycelial tissues. *Phytophthora sojae* isolates were cultured on cellophane (Ultra Clear Cellophane, RPI Crop) placed over 26% (v/v) V8 juice agar medium, and incubated at 25 °C for 7 days in darkness. The cellophane disks containing mycelia were then peeled off the agar medium, snap-frozen in liquid nitrogen and stored at -80 °C until used. Mycelial tissues were ground in liquid nitrogen to a fine powder. Total RNA was extracted using a solution of phenol-guanidine isothiocyanate (TRIzol) according to the instructions provided by the manufacturer (Invitrogen). The steps applied to extract RNA are as follows: 1) In each conical tube containing mycelial tissue (powdered form), TRIzol reagent (4 µL) was added immediately after the sample was removed from the liquid nitrogen, then the sample was mixed. 2) The suspension (1 mL) was dispensed into 2 ml Eppendorf tubes and 700 µL chloroform was added, the sample was vortexed, and kept at room temperature for 3 minutes. 3) Tubes were centrifuged at 14,000 rpm at 4 °C and the supernatant was collected into 15 mL conical tubes. 4) The supernatant was again dispensed into 1.5 mL Eppendorf tubes (≈500 µL each) and equal volume of isopropanol was added in each tube and kept at room temperature for 15 minutes. 5) The samples were centrifuged at 14000 rpm for 30 minutes at 4 °C. 6) The supernatant was discarded and pellet was washed with 1 ml 75% ethanol prepared in DEPC treated water, then centrifuged at 14000 rpm for 3 minutes at 4 °C. 7) The RNA pellet in the tube was air dried and dissolved in DEPC treated water and stored at -80 °C. Total RNA samples were quantified using a NanoDrop spectrophotometer (Thermo Scientific, USA) and their

integrity was checked by separating 200 ng of each sample on 1% (w/v) agarose gel in 1X TAE buffer (40 mM Tris, 40 mM acetate, 1 mM EDTA, pH 8.2-8.4). For RT-PCR, 1 µg of total RNA was treated with DNase I and first-strand cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) as per the manufacturer's directions. The *Avr3a* mRNA transcript was detected by PCR with the forward primer (5'-GCTGCTTCC TTCCTGGTTGC-3') and the reverse primer (5'-GCTGCTGCCTTTTGCTTCTC-3'). List of primers used in this project is given in table 2.2. Primers-specific for *P. sojae* *Actin* were used as a control. Biological and technical replicates of the RT-PCR were performed.

2.10 Virulence assay

To perform the virulence assay of *P. sojae* against *Rps3a*, soybean cultivars Williams (*rps*) and Williams isoline L83-570 (*Rps3a*) were used. For each isolate, 30 seeds of Williams and 30 seeds of L83-570 were sown in 10 cm pots (15 seeds in each pot). Soybean plants were grown for 7 days in a growth chamber which was maintained with 16 hours continuous light supply, 25 °C day temperature followed by 16 °C night temperatures before inoculations. *Phytophthora sojae* cultures were grown on 0.9% (w/v) V8 juice agar medium for 7 days, and macerated using 10 mL syringe with 18-gauge needle. The mycelial slurry (approximately 300 µL) was inoculated in the hypocotyl of soybean plant by making an incision below the epidermal layer (Figure 2.5). Plants were covered with plastic bags for 3 days, and then left for another 3 days without bags and the disease outcome was scored on day 6.

2.11 Identification of maternal parent in *P. sojae* cross P6497 X ACR10

To identify the maternal parent of F₁ hybrids from the cross P6497 X ACR10, a mitochondrial DNA marker was developed to track the inheritance of this organelle. Polymorphisms in the mitochondrial DNA sequence were identified by comparison of the reference genome P6497 to the re-sequenced strain ACR10, and verified by PCR amplification and Sanger sequencing. Specifically, primers were designed to flank an insertion/deletion polymorphisms of 96 bp, present in ACR10 but absent from P6497. The PCR reaction was performed using total genomic DNA as template and mitochondrial DNA sequence specific primers (forward primer 5'-TTTGGTGTATAGTTTCCCAACC-3', and reverse primer 5'-CGTGTTACTCACCCGTTCG-3'). The amplicon was then visualized by gel electrophoresis using 2% agarose gel. Maternal parentage of progeny was determined by comparing the size of the amplified product to that of control samples from *P. sojae* strains P6497 and ACR10.

2.12 Quantitative real time PCR (qRT-PCR) to study the expression level of *Avr3a* gene among *P. sojae* hybrids

To quantitatively measure the expression of *Avr3a* in selected *P. sojae* cultures, qRT-PCR was performed. To perform qRT-PCR, PerfeCTa®SYBR® Green SuperMix (Quanta Biosciences) was used with CFX96 real-time PCR detection system (Bio-Rad Laboratories, Inc., USA). The cDNA was prepared from RNA samples and purified as described above, using Superscript III reverse transcriptase. The *Avr3a* gene expression was analysed using the gene specific primers (forward primer: 5'-

TCGCTCAAGTTGTGG TCGTC-3' and reverse primer: 5'-TCGACAGCGTCCTATCTTCG-3'). The primers used for reference gene (*Actin*) amplification were forward primer: 5'-CGAAATTGT GCGCG ACATCAAG-3' and reverse primer: 5'-GGTACCGCCC GACAGCACGAT-3'. The data were analyzed using a CFX manager (Bio-Rad Laboratories Inc., USA).



Figure 2.5 Photograph showing inoculum of *P. sojae* in the soybean hypocotyl. For the virulence assay, inoculum slurry of *P. sojae* culture (mycelia) on V8 juice medium was prepared using 10 mL syringe. The inoculum (*P. sojae* mycelia + V8 juice medium) was prepared and applied to plants by making a small incision on hypocotyl of one week old soybean seedlings.

Table 2.3 Oligonucleotide Primers used in this study

Target gene/ marker	Primer name	5' to 3' sequence	Applications
<i>Avr3a</i>	<i>Sp92-33F</i>	GCTGCTTCCTTCCTGGTT GC	PCR of genomic DNA
	<i>Sp92-325R</i>	GCTGCTGCCTTTTGCTTCTC	(Hybrid identification) RT-PCR transcript detection (Qutob et al., 2013)
<i>Avr3a</i>	<i>Avr3a-qRT-F</i>	TCGCTCAAGTTGTGGTCGTC	Quantitative real time PCR
	<i>Avr3a-qRT-R</i>	TCGACAGCGTCCTATCTTCG	(Dong et al., 2011b)
<i>Avh320</i>	<i>Avh320-87-F</i>	AACGCTCTCGAAAGTGGC	PCR of genomic DNA (Hybrid identification)
	<i>Avh320-19-R</i>	AAAGAACTTCGACAGCC	(Designed by using Primer 3 software)
<i>Scaf-29-M2</i>	<i>Scaf-29-M2-F</i>	CCCTCGAGAACGCCAACTT	PCR of genomic DNA (Hybrid identification)
	<i>Scaf-29-M2-R</i>	CCTCGCTCGCCTTCATCC	(Designed by using Primer 3 software)
<i>Actin</i>	<i>7-2g-MY(ActinA)-F</i>	CGAAATTGTGCGCGACATCAAG	RT-PCR and Quantitative real time PCR
	<i>7-2g-MY-(ActinA)-R</i>	GGTACCGCCCGACAGCACGAT	(housekeeping gene) (Qutob et al., 2013)
Mitochondrial Sequence	<i>mt1-P5008-F</i>	TTTGGTGTATAGTTCCCAACC	Identify maternal parent
	<i>mt1-P5008-R</i>	CGTGTTACTCACCCGTTTCG	(Designed by using Primer 3 software)

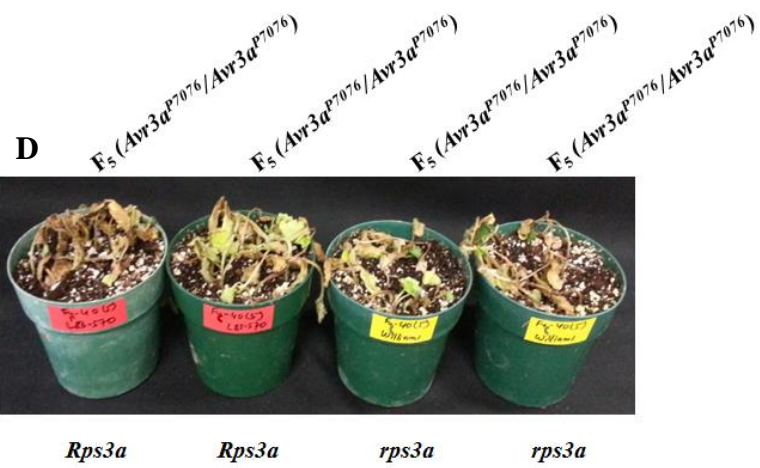
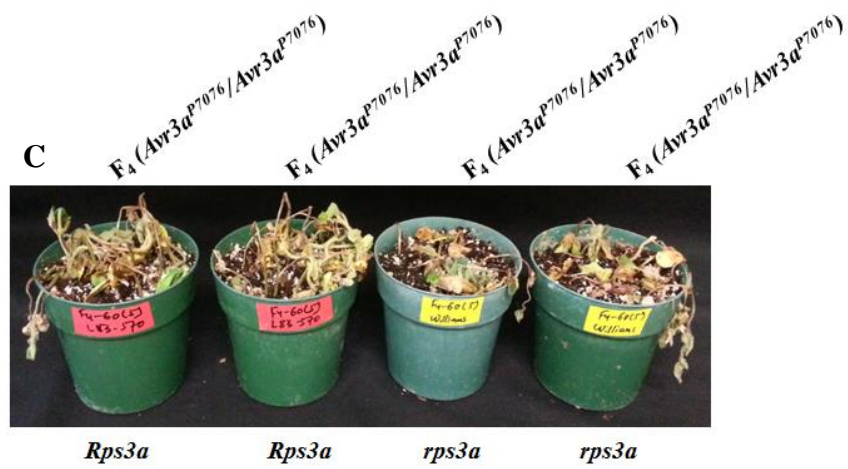
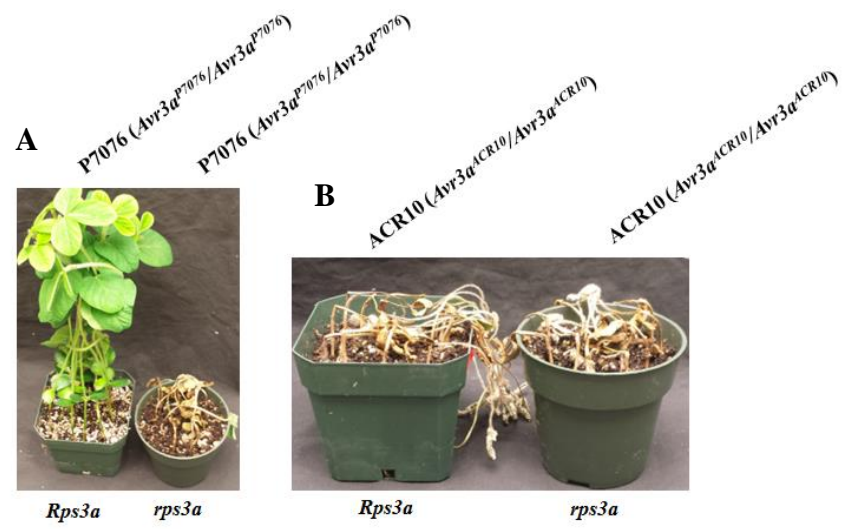
Chapter 3: Results

3.1 Transgenerational gene silencing of *Avr3a* is meiotically stable over multiple generations in progeny from a cross of ACR10 X P7076

To study the meiotic stability of gene silencing, F₄ progeny were developed by self-fertilization of four different F₃ (*Avr3a*^{P7076}/*Avr3a*^{P7076}) progeny. The four different F₃ (F₃-40, F₃-60, F₃-83, and F₃-87) isolates were selected randomly from progeny with the genotype *Avr3a*^{P7076}/*Avr3a*^{P7076}, and self-fertilized to produce F₄ progeny. From each of the four F₃ progeny, four germinating oospores were isolated to develop F₄ progeny. Therefore a total of 16 F₄ progeny were generated. Virulence assays were performed on test (*Rps3a*) and control (*rps3a*) soybean plants, and the cultures were tested for the presence of *Avr3a* mRNA transcript by RT-PCR. The virulence assays show that 15/16 of the progeny are virulent towards soybeans carrying the *Rps3a* gene, but one F₄ lost general virulence or pathogenicity, being avirulent to both test (*Rps3a*) and control (*rps3a*) plants (Figure 3.1 C, E, Table 3.1). There are no detectable *Avr3a* mRNA transcripts among all (16/16) tested F₄ progeny including the avirulent individual (Figure 3.2 A).

Similarly, to develop F₅ progeny, one individual from each of the four different F₄ strains was selected and processed for self-fertilization. The isolate that had lost general virulence was excluded, but otherwise the four individuals were selected randomly. From each F₄ self-fertilization, four oospores were isolated and developed into F₅ progeny, thus making a total number of sixteen F₅ progeny. Virulence assays were performed on test (*Rps3a*) and control (*rps3a*) soybean plants, and the cultures were tested for the presence

of *Avr3a* transcript by RT-PCR. The virulence assay results show that 15/16 of the F₅ progeny are virulent towards soybeans carrying the *Rps3a* gene, but one F₅ lost general virulence or pathogenicity, since it is avirulent to both test (*Rps3a*) and control (*rps3a*) plants (Figure 3.1 D, Table 3.2). There are no detectable *Avr3a* mRNA transcripts among all (16/16) tested F₅ progeny (Figure 3.2 B). Although one isolate in each F₄ and F₅ progeny lost their general virulence towards soybean plants with or without *Rps3a*, it was not due to expression of the *Avr3a* transcript. Therefore, *Avr3a* gene silencing was stable in all F₄ and F₅ progeny.



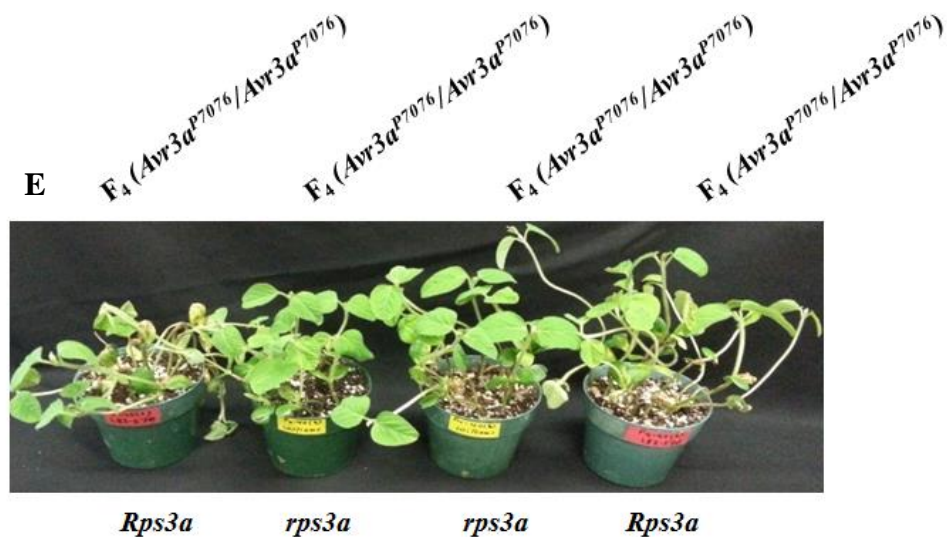


Figure 3.1 Photographs of disease outcomes in soybean plants infected with *P. sojae* parental strains, F₄ and F₅ progeny. Inoculum of *P. sojae* mycelia was prepared after 7 days incubation at 25°C on 0.9% (w/v) V8 juice agar medium. Soybean cultivar Williams (*rps3a*) and the isolate L83-570 (*Rps3a*) were inoculated with *P. sojae* mycelia into the hypocotyl and photographs taken five days later. (A) *P. sojae* parental strains, P7076 (*Avr3a*^{P7076}/*Avr3a*^{P7076}). (B) *P. sojae* parental strain ACR10 (*Avr3a*^{ACR10}/*Avr3a*^{ACR10}). *P. sojae* P7076 (*Avr3a*^{P7076}/*Avr3a*^{P7076}) is avirulent towards *Rps3a* whereas ACR10 (*Avr3a*^{ACR10}/*Avr3a*^{ACR10}) is virulent. (C) A representative F₄ progeny (*Avr3a*^{P7076}/*Avr3a*^{P7076}) showing virulence towards soybean plants carrying *Rps3a* gene. (D) A representative F₅ progeny showing virulence towards soybean plants carrying *Rps3a* gene. (E) An F₄ generation individual showing loss of virulence to test (*Rps3a*) and control (*rps3a*) plants.

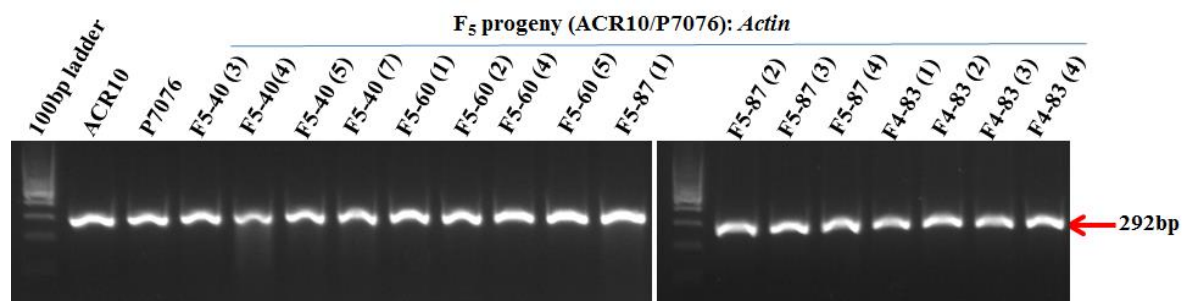
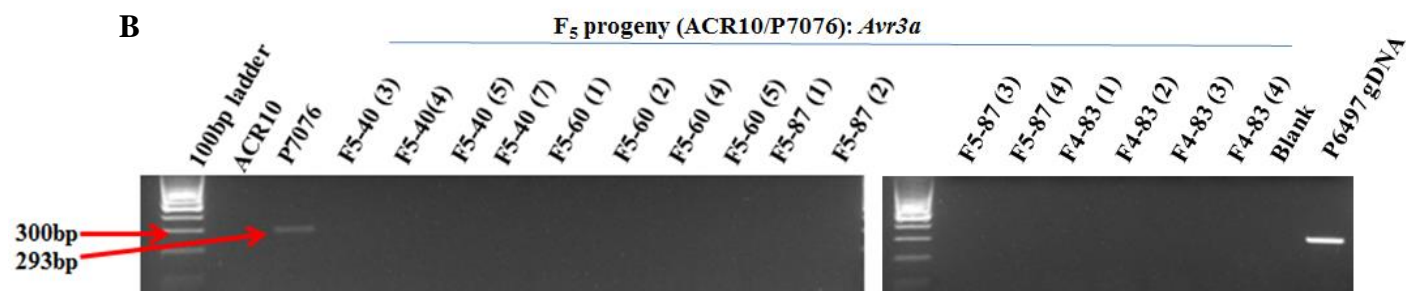
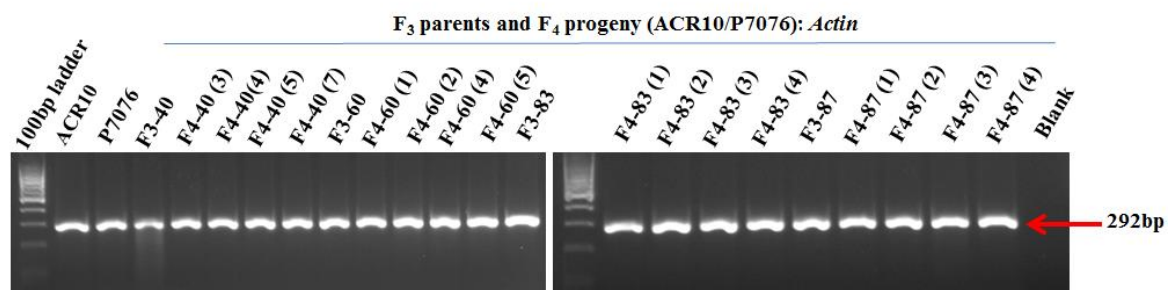
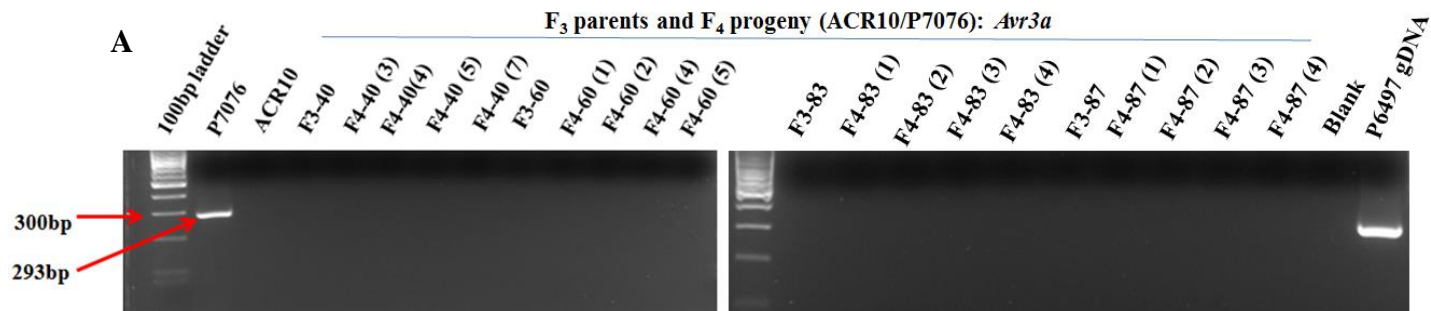


Figure 3.2 RT-PCR analyses of F₄ and F₅ progeny for the *Avr3a* mRNA transcript.

Shown are photographs of agarose gels after staining DNA with a fluorescent dye. The F₄ and F₅ progeny were developed from a cross of ACR10 X P7076. Total RNA was extracted from *P. sojae* mycelia and reverse transcribed into cDNA. RT-PCR was carried out using *Avr3a* gene specific primers (*Sp92-33* F and *Sp92-325* R) and was compared with the parental strains. The size of amplicon is 293 bp. A sample of genomic DNA of strain P6497 provided a positive control for PCR, while a PCR mixture without any DNA sample input (blank) provided a negative control. *Phytophthora sojae* *Actin* gene was used as a control to amplify *Actin*, forward primer *7-2g-MY(Actin A)-F* and reverse primer *7-2g-MY(Actin A)-R* was used; the size of *Actin* amplicon is 292 bp. (A) RT-PCR of *Avr3a* and *Actin* transcripts in F₄ progeny. (B) RT-PCR of *Avr3a* and *Actin* transcripts in F₅ progeny.

Table 3.1 Virulence outcomes and *Avr3a* transcript detection in F₄ progeny

F ₃ parents and F ₄ progeny of ACR10 X P7076	Virulence on L83-570 (<i>Rps3a</i>) ¹		Virulence on Williams (<i>rps3a</i>)		<i>Avr3a</i> mRNA transcript (RT-PCR)
	Number of dead plants /60	Assigned phenotype	Number of dead plants /60	Assigned phenotype	
F ₃ -40	60	V	60	V	Negative
F ₄ -40 (3)	9	A	15	A	Negative
F ₄ -40(4)	43	V	45	V	Negative
F ₄ -40(5)	60	V	60	V	Negative
F ₄ -40 (7)	60	V	60	V	Negative
F ₃ -60	60	V	60	V	Negative
F ₄ -60 (1)	60	V	60	V	Negative
F ₄ -60 (2)	55	V	60	V	Negative
F ₄ -60 (4)	53	V	56	V	Negative
F ₄ -60 (5)	60	V	60	V	Negative
F ₃ -87	58	V	60	V	Negative
F ₄ -87 (1)	60	V	60	V	Negative
F ₄ -87 (2)	55	V	53	V	Negative
F ₄ -87 (3)	60	V	60	V	Negative
F ₄ -87 (4)	60	V	60	V	Negative
F ₃ -83	60	V	60	V	Negative
F ₄ -83 (1)	60	V	60	V	Negative
F ₄ -83 (2)	60	V	60	V	Negative
F ₄ -83 (3)	60	V	60	V	Negative
F ₄ -83(4)	59	V	60	V	Negative

¹Virulence assays consisted of two replicates of 30 plants each, for test (*Rps3a*) and control (*rps3a*) plants. A, avirulent; V, virulent.

Table 3.2 Virulence outcomes and *Avr3a* transcript detection in F₅ progeny

F ₄ parents and F ₅ progeny of ACR10 X P7076	Virulence on L83-570 (<i>Rps3a</i>) ¹		Virulence on Williams (<i>rps3a</i>)		<i>Avr3a</i> mRNA transcript (RT-PCR)
	Number of dead plants /60	Assigned phenotype	Number of dead plants /60	Assigned phenotype	
F ₄ -40 (7)	60	V	60	V	Negative
F ₅ -40 (1)	20	A	12	A	Negative
F ₅ -40(2)	59	V	57	V	Negative
F ₅ -40(3)	60	V	59	V	Negative
F ₅ -40 (4)	60	V	60	V	Negative
F ₄ -60 (5)	60	V	60	V	Negative
F ₅ -60 (1)	60	V	60	V	Negative
F ₅ -60 (2)	53	V	52	V	Negative
F ₅ -60 (3)	55	V	56	V	Negative
F ₅ -60 (4)	54	V	47	V	Negative
F ₄ -87 (1)	60	V	60	V	Negative
F ₅ -87 (1)	60	V	60	V	Negative
F ₅ -87 (2)	56	V	58	V	Negative
F ₅ -87 (3)	60	V	60	V	Negative
F ₅ -87 (4)	45	V	46	V	Negative
F ₄ -83 (3)	60	V	60	V	Negative
F ₅ -83 (1)	60	V	60	V	Negative
F ₅ -83 (2)	60	V	60	V	Negative
F ₅ -83 (3)	60	V	60	V	Negative
F ₅ -83(4)	60	V	60	V	Negative

¹Virulence assays consisted of two replicates of 30 plants each, for test (*Rps3a*) and control (*rps3a*) plants. V-virulent; A-avirulent

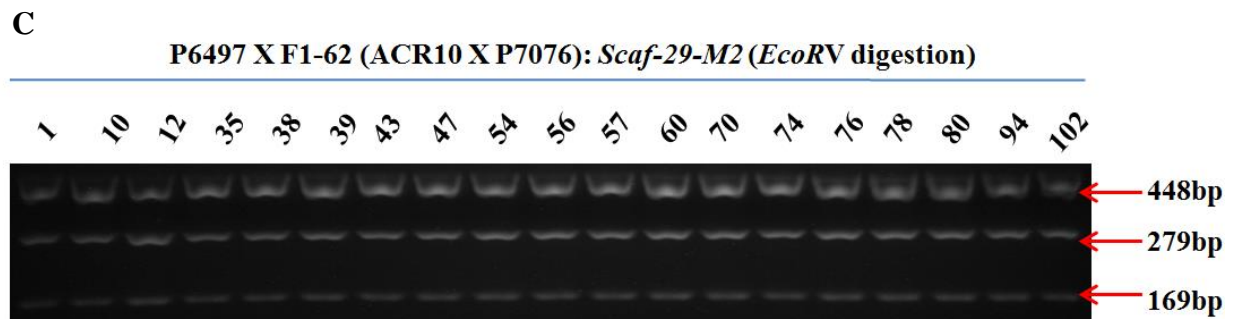
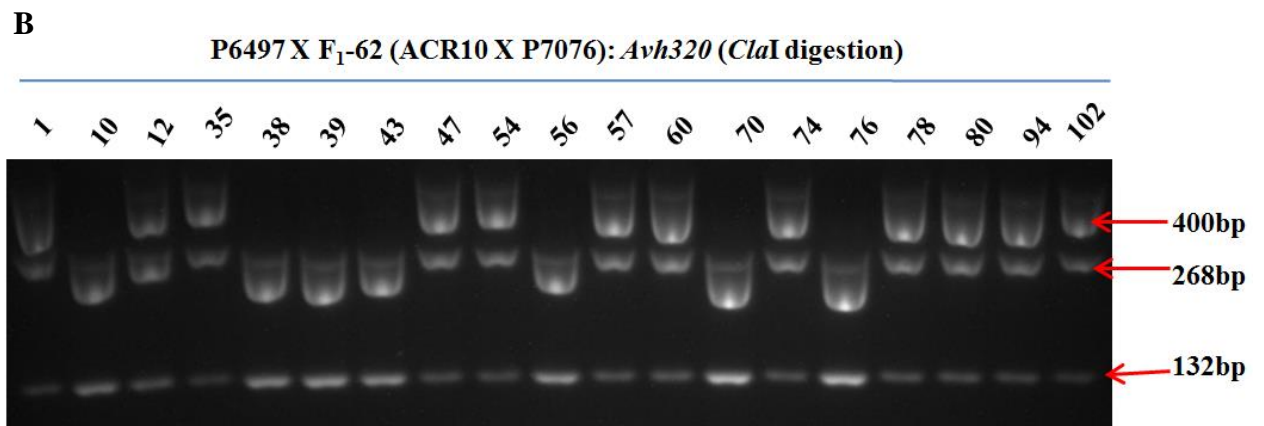
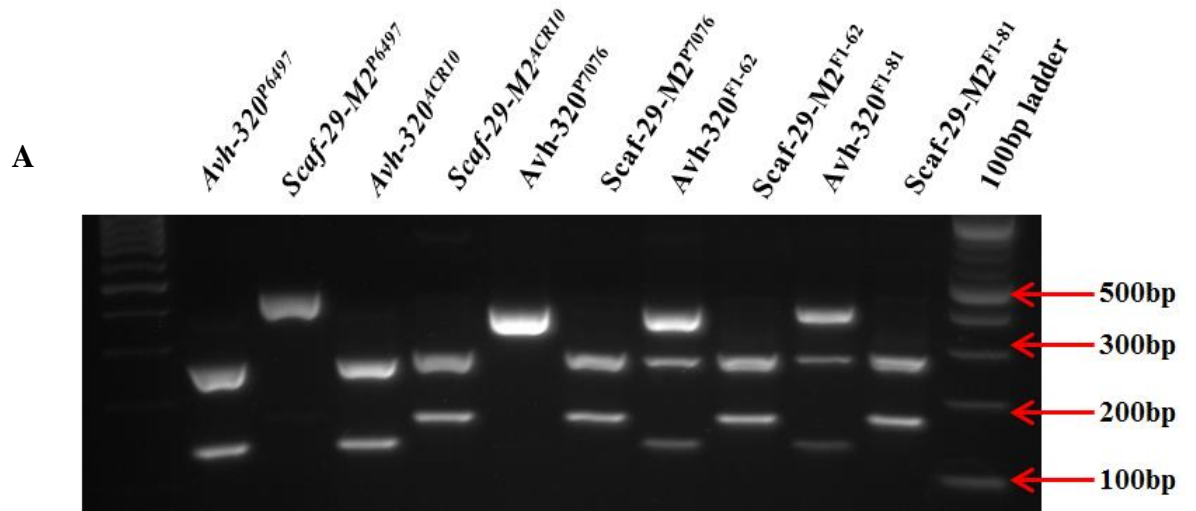
3.2 Gene silencing of *Avr3a* is released by out-crossing

To determine whether silenced alleles of *Avr3a*^{P7076} have the capacity to silence expressed alleles of other strains of *P. sojae*, a test cross was performed between *P. sojae* P6497 X F₁ (ACR10 X P7076). A total of 110 oospores were isolated from a cross of P6497 X F₁-62 (ACR10 X P7076). Due to the identical *Avr3a* sequence of P6497 and ACR10, hybrids of interest (*Avr3a*^{P6497}/*Avr3a*^{P7076} or *Avr3a*^{P6497}/*Avr3a*^{ACR10}) were identified using three different co-dominant CAPs markers. The marker *Avh320* is linked to *Avr3a* locus (Figure 3.3 A, B, C, D, E). A total of 14 *Avr3a*^{P6497}/*Avr3a*^{P7076} and 17 *Avr3a*^{P6497}/*Avr3a*^{ACR10} hybrids were identified from the 110 progeny of this cross (Figure 3.3 D). Virulence testing of the 31 hybrids indicated that 30 are avirulent towards test (*Rps3a*) plants, whereas one (culture #12; *Avr3a*^{P6497}/*Avr3a*^{ACR10}) is virulent (Figure 3.5 A, Table 3.3). All 31 isolates are virulent towards control (*rps3a*) plants. Analysis of *Avr3a* mRNA levels by RT-PCR showed that all 31 cultures produce *Avr3a* transcripts. However, the *Avr3a* mRNA is barely detectable in the culture (#12) that is virulent on *Rps3a* plants (Figure 3.6 A, B).

This experiment was replicated using another F₁ individual. The cross of *P. sojae* P6497 X F₁-81(ACR10 X P7076) was performed and a total of 19 hybrids of interest were identified; 6/19 were *Avr3a*^{P6497}/*Avr3a*^{ACR10} and 13/19 were *Avr3a*^{P6497}/*Avr3a*^{P7076} (Figure 3.3 E, 3.4 B). Analysis for *Avr3a* transcripts by RT-PCR showed that 18/19 of the hybrids possess *Avr3a* mRNA, whereas it is not detectable in the remaining (virulent) culture (Figure 3.6 C). Among the 18 individuals with detectable *Avr3a* transcript, the signal intensity of *Avr3a* amplification product appears to be variable (Figure 3.6 C).

Likewise, from virulence assays it was found that the phenotypic penetrance of the avirulence trait in several isolates was incomplete because not all plants with *Rps3a* gene were killed (Figure 3.5 B, Table 3.4). To more accurately measure the *Avr3a* gene expression level among the cultures, quantitative (q) RT-PCR was performed. Comparison of the results from qRT-PCR analysis and virulence tests demonstrates a correlation ($R^2 = 0.44$, $p = 0.0015$), with higher expression levels of *Avr3a* leading to greater phenotypic penetrance of the avirulence trait (Figure 3.7).

These experiments show that the *Avr3a*^{P6497} allele is not paramutable. The establishment and propagation of *Avr3a* silencing is strain specific and silencing could be conditional upon epistatic factors or allele-specific imprinting.



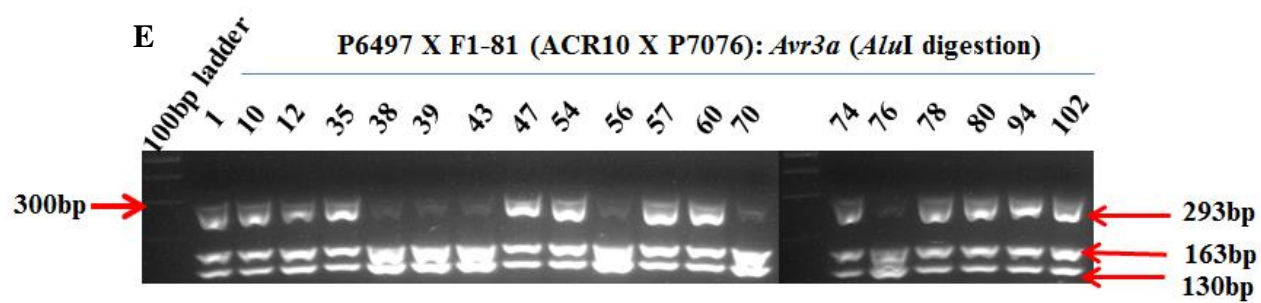
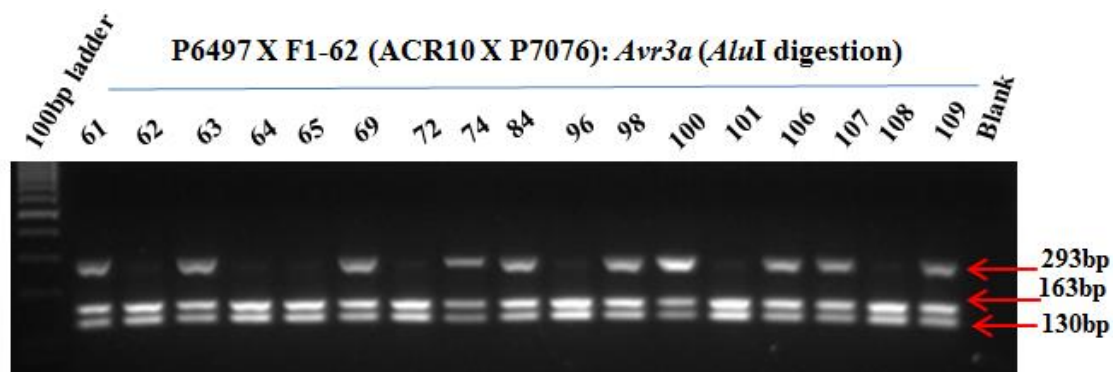
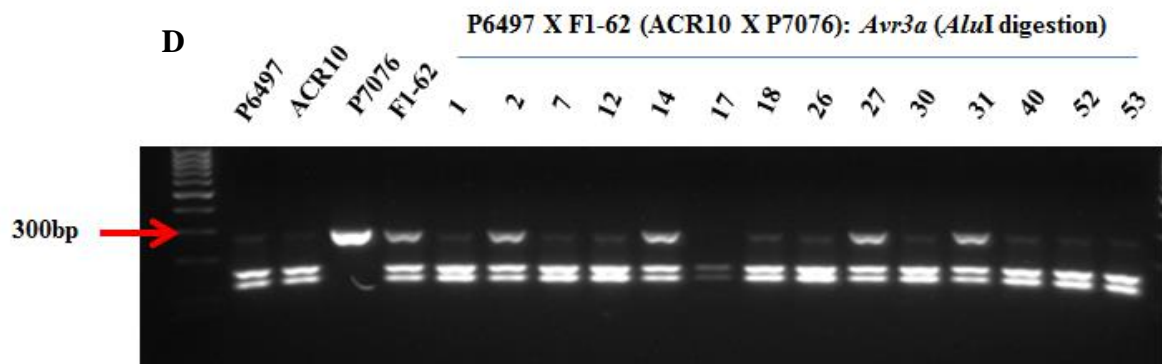


Figure 3.3 DNA marker analysis to identify hybrid cultures from a cross of *P. sojae* P6497 X F₁ (ACR10 X P7076). Shown are photographs of fluorescently stained (SYBR safe) 2.5% agarose gels illustrating cleaved amplified polymorphic (CAP) analyses of P6497, ACR10, and P7076 alleles from *P. sojae* isolates. The DNA marker *Avh320* was used to distinguish alleles of P7076 from P6497 and ACR10. The *P. sojae* *Avh320*^{P6497} and *Avh320*^{ACR10} alleles possess a restriction site for *Cla*I, but *Avh320*^{P7076} does not. Therefore CAPs analysis for *Avh320* in the P6497 and ACR10 alleles results in two bands, whereas analysis of the P7076 allele results in a single band. The DNA marker *Scaf-29-M2* was used to distinguish alleles of P6497 from ACR10 and P7076. The *P. sojae* *Scaf-29-M2*^{ACR10} and *Scaf-29-M2*^{P7076} alleles have a restriction site for *Eco*RV, but the *Scaf-29-M2*^{P6497} allele does not, therefore CAPs analysis of the ACR10 and P7076 allele results in two bands whereas analysis of the P6497 allele results in a single band. Strains that carry heterozygous alleles for each of the markers will possess three bands by CAPs analysis. **(A)** A CAPs analysis of DNA markers *Avh320* and *Scaf-29-M2* in the parental strains P6497, ACR10, and P7076, and in the F₁ hybrids F₁-62 and F₁-81. **(B)** A CAPs analysis of hybrids obtained from the test cross P6497 X F₁-62 (ACR10 X P7076) using the DNA marker *Avh320*. **(C)** A CAPs analysis of hybrids obtained from the test cross P6497 X F₁-62 (ACR10 X P7076) using the DNA marker *Scaf-29-M2*. **(D)** A CAPs analysis of the parental strains (P6497, ACR10, and P7076) and hybrids obtained from the test-cross of P6497 X F₁-62 (ACR10 X P7076) using DNA marker *Avr3a*. The *Avr3a* alleles from strains P6497 and ACR10 possess a restriction site for *Alu*I but the P7076 allele does not. Therefore *Avr3a* CAPs analysis of hybrid cultures from the test cross P6497 X F₁ (ACR10 X P7076) results in two bands for

Avr3a^{P6497}/*Avr3a*^{ACR10} genotypes and three bands for *Avr3a*^{P6497}/*Avr3a*^{P7076} genotypes. (E)

A CAPs analysis of the *Avr3a* gene in hybrids obtained from the test-cross P6497 X F₁-81(ACR10 X P7076). To perform PCR, marker specific primers were used (Table 2.2). For *Avh320* marker, forward primer *Avh320-87-F* and reverse primer *Avh320-19-R* were used; for *Scaf-29-M2*, forward primer *Scaf-29-M2-F* and reverse primer *Scaf-29-M2-R* were used; and for *Avr3a*, forward primer *Sp92-33F* and reverse primer *Sp92-325R* were used to perform PCR reaction. The sizes of the DNA fragments are indicated. Individual numbers represent hybrid isolates.

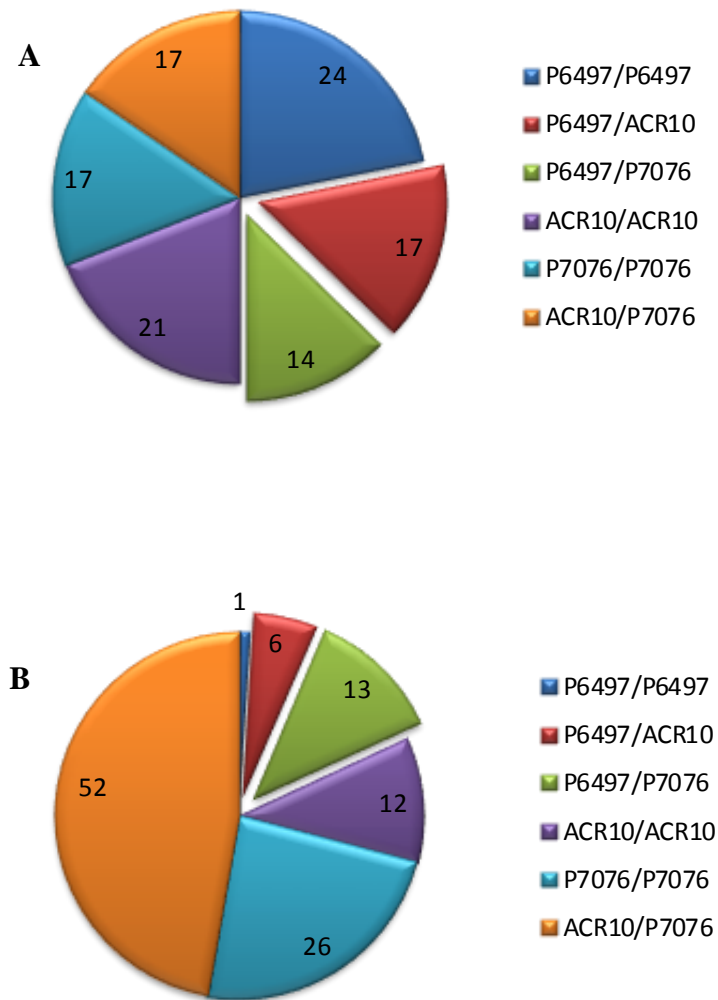


Figure 3.4 Deduced *Avr3a* genotypes of the progeny isolated from test cross of P6497 X F₁ (ACR10 X P7076). Hybrids of the predicted genotype of $Avr3a^{P6497}/Avr3a^{ACR10}$ and $Avr3a^{P6497}/Avr3a^{P7076}$ were selected for virulence assays and RT-PCR analysis. **(A)** *Avr3a* genotypes of offspring from test cross P6497 X F₁-62 (ACR10 X P7076). **(B)** *Avr3a* genotypes of offspring from test cross P6497 X F₁-81(ACR10 X P7076).

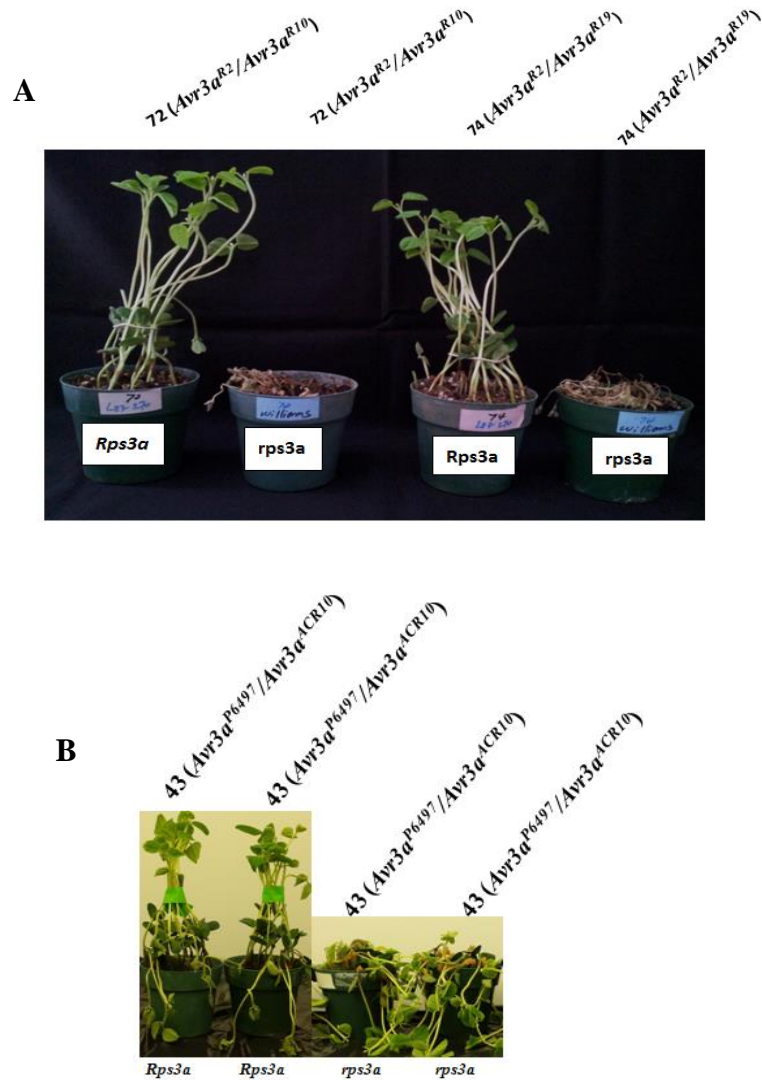
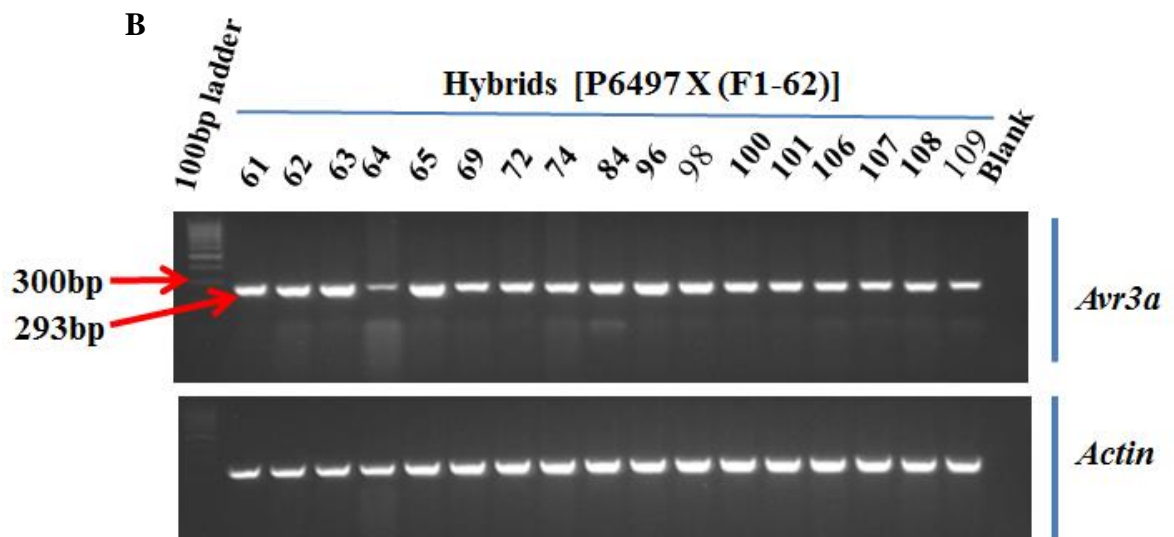
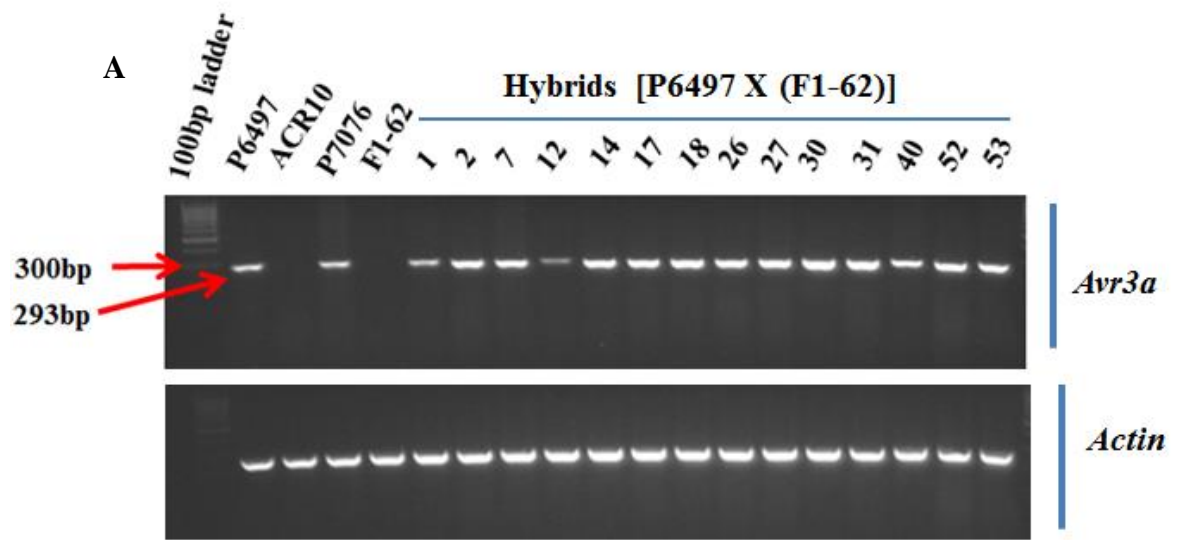


Figure 3.5 Photographs illustrating the outcomes of virulence assays of hybrids isolated from test cross of P6497 X F₁ (ACR10 X P7076). Shown are the results of inoculation of progeny #72 ($Avr3a^{P6497}/Avr3a^{ACR10}$) and #74 ($Avr3a^{P6497}/Avr3a^{P7076}$) developed by test cross of P6497 X F₁-62 (ACR10 X P7076) on soybean isolate L83-570 (*Rps3a*) and Williams (*rps3a*). **(B)** Example of incomplete penetrance of *Avr3a* avirulence trait. Shown are the results of inoculation of progeny #43 ($Avr3a^{P6497}/Avr3a^{ACR10}$) developed by test cross of P6497 X F₁-81 (ACR10 X P7076) on soybean isolines L83-570 (*Rps3a*) and Williams (*rps3a*).



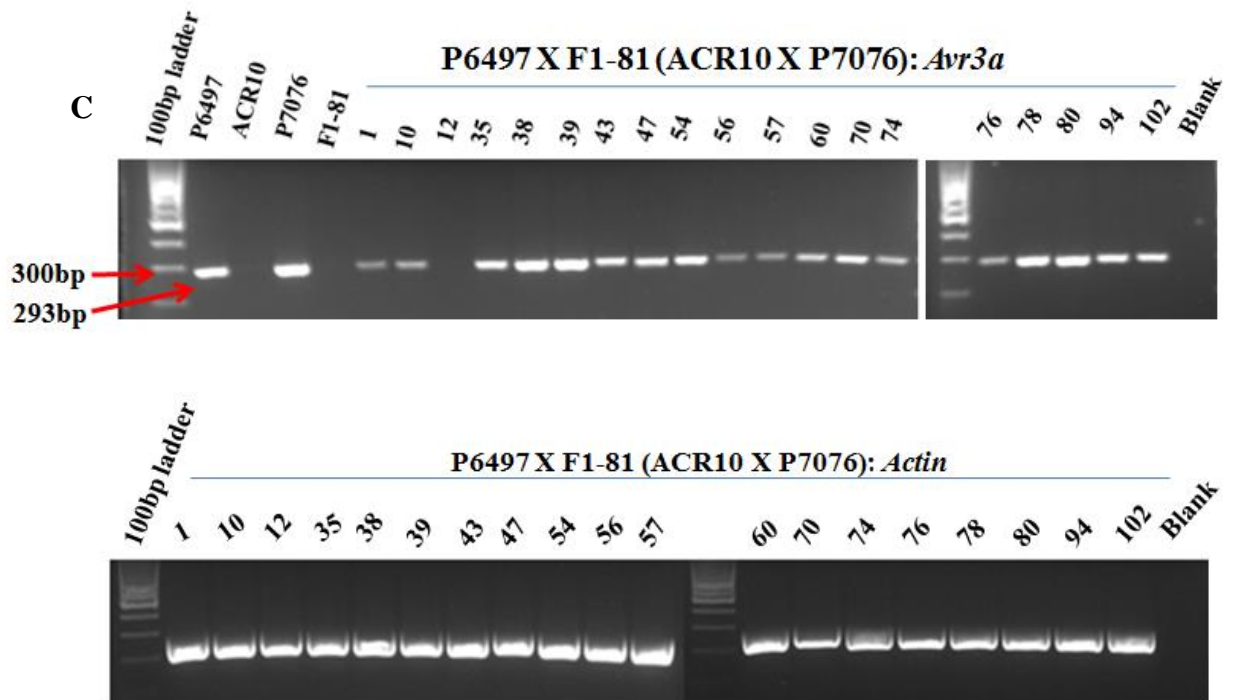


Figure 3.6 Analysis for *Avr3a* transcripts in parental strains and progeny of test-cross P6497 X F₁ (ACR10 X P7076). The cDNA was synthesized using total RNA from the mycelia of *P. sojae*. RT-PCR was carried out using *Avr3a* gene-specific forward primer *Sp92-33F* and reverse primer *Sp92-325R*. *Phytophthora sojae* *Actin* gene was used as a control to amplify *Actin*, forward primer *7-2g-MY(Actin A)-F* and reverse primer *7-2g-MY(Actin A)-R* was used. The size of *Avr3a* amplicon is 293 bp, gDNA is the genomic DNA of *P. sojae* P6497. (A, B) RT-PCR analysis of *Avr3a* mRNA transcript and *Actin* of hybrids isolated from testcross of P6497 X F₁- 62 (ACR10 X P7076). (C) RT-PCR analysis of *Avr3a* mRNA transcript and *Actin* of hybrids isolated from test-cross P6497 X F₁-81(ACR10 X P7076).

Table 3.3 Virulence outcomes and *Avr3a* transcript detection in progeny of P6497 X F₁-62 (ACR10/P7076)

P6497 X F ₁ -62 hybrids	Virulence on L83-570 (<i>Rps3a</i>) ¹		Virulence on Williams (<i>rps3a</i>)		<i>Avr3a</i> mRNA transcript by RT-PCR
	Number of dead plants /60	Assigned phenotype	Number of dead plants /60	Assigned phenotype	
1. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	0	A	60	V	Positive
2. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	0	A	60	V	Positive
7. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	0	A	60	V	Positive
12. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	60	V	60	V	Weak positive
14. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	1	A	60	V	Positive
17. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	0	A	60	V	Positive
18. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	0	A	60	V	Positive
27. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	2	A	60	V	Positive
30. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	0	A	60	V	Positive
31. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	0	A	60	V	Positive
40. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	0	A	60	V	Positive
51. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	0	A	60	V	Positive
52. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	0	A	58	V	Positive
53. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	0	A	60	V	Positive
61. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	0	A	0	A	Positive
62. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	0	A	60	V	Positive
63. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	0	A	55	V	Positive
64. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	0	A	56	A	Positive
65. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	0	A	60	V	Positive
69. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	0	A	60	V	Positive
72. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	0	A	60	V	Positive
74. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	0	A	60	V	Positive
84. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	0	A	59	V	Positive
96. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	0	A	60	V	Positive
98. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	0	A	60	V	Positive
100. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	0	A	60	V	Positive
101. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	0	A	60	V	Positive
106. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	0	A	60	V	Positive
107. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	0	A	60	V	Positive
108. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	0	A	60	V	Positive
109. (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	0	A	60	V	Positive

¹Virulence assays consisted of two replicates of 30 plants each, A -avirulent, V- virulent

Table 3.4 Virulence outcomes and *Avr3a* transcript detection in progeny of P6497 X F₁-81(ACR10/P7076)

P6497 X F ₁ -81	Virulence on L83-570 (<i>Rps3a</i>) ¹		Virulence on Williams (<i>rps3a</i>)		<i>Avr3a</i> mRNA transcript by RT-PCR
	Number of dead plants /60	Assigned phenotype	Number of dead plants /60	Assigned phenotype	
1 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	33	A'	60	V	Positive
10 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	24	A'	60	V	Positive
12 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	60	V	60	V	Negative
35 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	4	A	60	V	Positive
38 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	6	A	60	V	Positive
39 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	5	A	60	V	Positive
43 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	12	A'	60	V	Positive
47 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	2	A	60	V	Positive
54 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	8	A	60	V	Positive
56 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	10	A'	60	V	Positive
57 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	11	A'	60	V	Positive
60 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	13	A'	60	V	Positive
70 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	3	A	60	V	Positive
74 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	4	A	60	V	Positive
76 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{ACR10})	8	A	60	V	Positive
78 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	0	A	60	V	Positive
80 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	0	A	60	V	Positive
94 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	0	A	60	V	Positive
102 (<i>Avr3a</i> ^{P6497} / <i>Avr3a</i> ^{P7076})	9	A	60	V	Positive

¹Virulence assays consisted of two replicates of 30 plants each, for test (*Rps3a*) and control (*rps3a*) plants A -avirulent, V- virulent. A' - incomplete avirulent

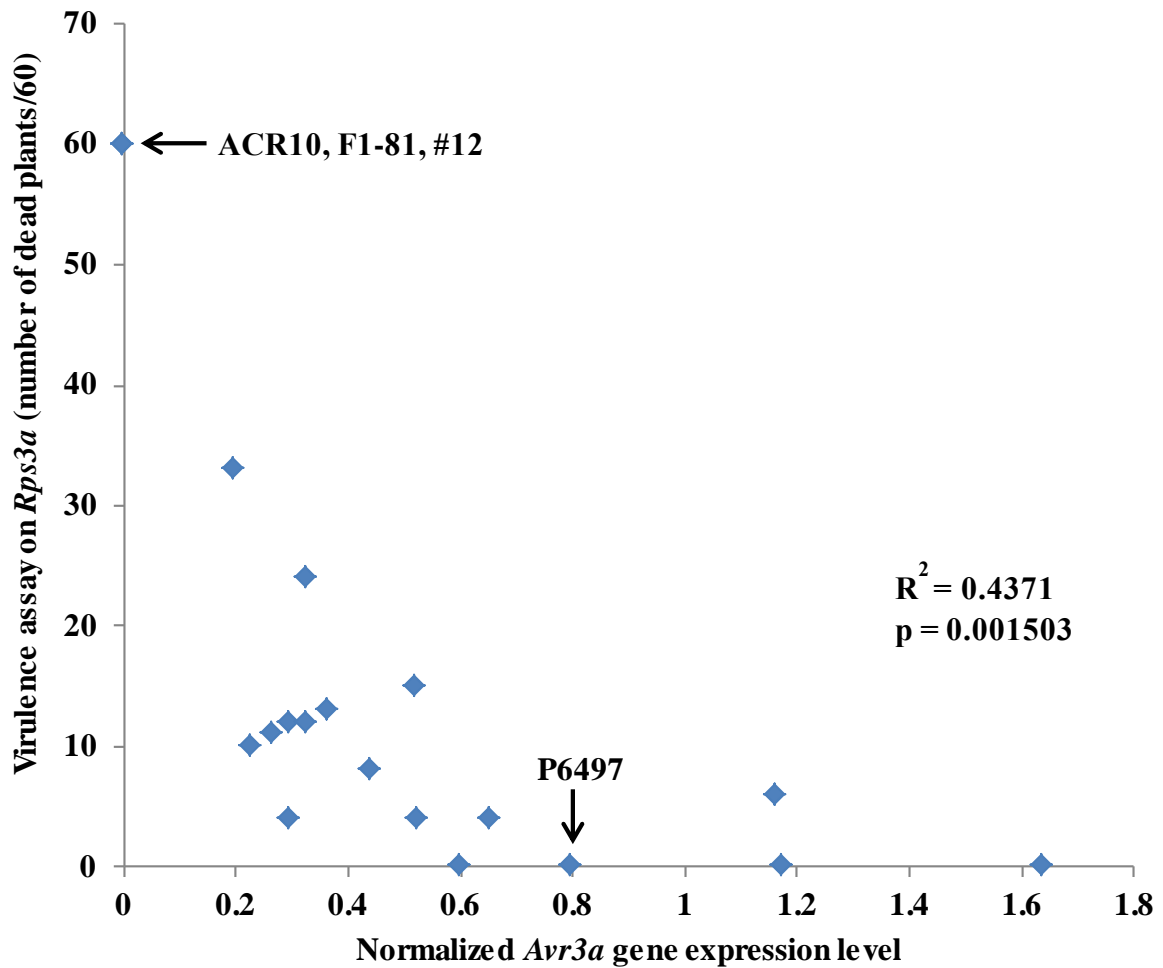


Figure 3.7 Relative expression profile of *Avr3a* gene among the hybrids obtained from P6497 X F₁-81 test-cross vs. virulence assay on soybean *Rps3a*. Each point is a mean from two biological replicates. Expression of *Avr3a* was normalized with *P. sojae Actin*. Parental strain P6497 was used as positive control and ACR10 (overlapped with other data on Y-axis) as negative control. On Y-axis, three data points (ACR10, F₁-81, and #12) exactly overlap. Regression analysis shows the correlation between two variables: *Avr3a* gene expression and virulence of the isolates with $R^2 = 0.4371$ ($p = 0.001503$).

3.3 Release of silencing of *Avr3a*^{P7076} in progeny from test-cross P6497 X F₁ (ACR10 X P7076)

In order to determine allele-specific expression of *Avr3a* in progeny from the test cross P6497 X F₁ (ACR10 X P7076), RT-PCR was performed on mRNA samples, using an *Avr3a* CAPs marker that distinguishes *Avr3a* alleles of P6497 and P7076. An allele-specific expression test is only feasible for progeny with the genotype *Avr3a*^{P6497}/*Avr3a*^{P7076}. As the DNA marker relies on sequence differences of *Avr3a* gene, it is not possible to determine whether there is allele-specific expression for progeny with the genotype *Avr3a*^{P6497}/*Avr3a*^{ACR10} since the two alleles are sequence identical. Results from this analysis show that the progeny with the genotype *Avr3a*^{P6497} / *Avr3a*^{P7076} produce transcripts of both alleles; thus the silenced *Avr3a* allele of P7076 was released when test-crossed with P6497 (Figure 3.8).

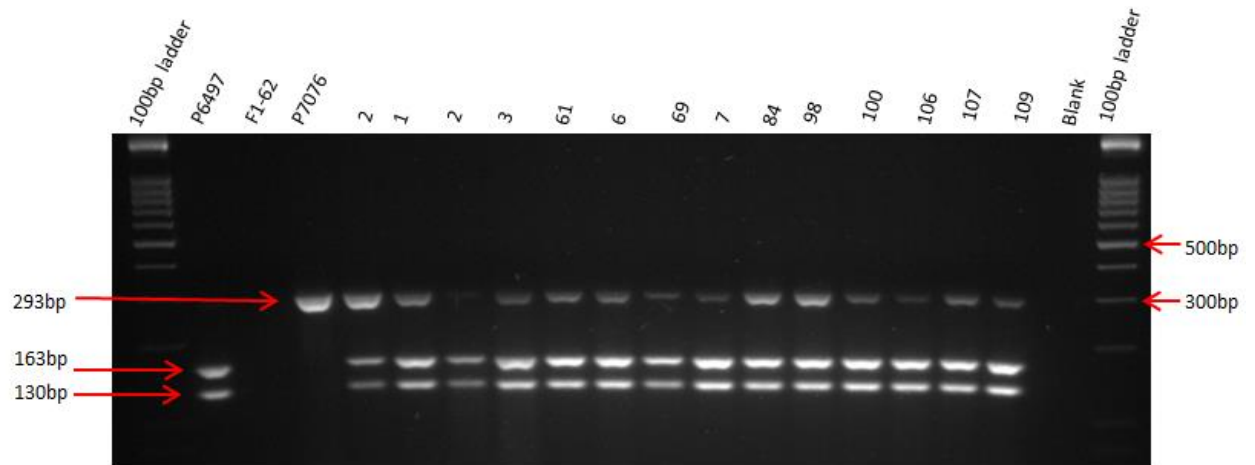


Figure 3.8 Analysis for allele-specific expression of *Avr3a*, illustrating the release of gene silencing of the P7076 allele in progeny from the test-cross P6497 X F₁ (ACR10 X P7076). An RT-PCR and CAPs analysis of *Avr3a* mRNA transcripts from hybrids with the genotype *Avr3a*^{P6497}/*Avr3a*^{P7076}. The RT-PCR was performed using *Avr3a* specific primers (forward primer *Sp92-33F* and reverse primer *Sp92-325R*) and the amplified product was digested with the restriction enzyme *AluI*. The *Avr3a*^{P6497} allele contains a restriction site for *AluI* but the *Avr3a*^{P7076} allele does not.

3.4 Crosses of *P. sojae* strains P6497 X ACR10 results in F₁ hybrids that segregate for virulence towards *Rps3a*

From the first out-cross, a total of 110 germinated oospores were isolated. However, DNA marker analysis demonstrated that most (109/110) of the progeny resulted from the self-fertilization of parental strain P6497; a single F₁ hybrid progeny was identified using the co-dominant DNA CAPs marker *Scaf-29-M2* (Figure 3.10 A). It is not possible to track directly the inheritance of *Avr3a* because this gene is sequence-identical in the two parental strains. The single hybrid isolate was tested for virulence towards *Rps3a* and for the presence of *Avr3a* mRNA transcript. Results indicate that the F₁ is avirulent towards *Rps3a* and positive for *Avr3a* mRNA transcript (Table 3.5).

The second attempt to produce hybrid progeny from P6497 X ACR10 was more successful. From a total of 110 oospores, 19 F₁ hybrids were identified (Figure 3.9). Of the remaining offspring, 57/110 and 34/110 resulted from self-fertilization of P6497 and ACR10 respectively (Figure 3.10). Results from the RT-PCR analysis of *Avr3a* mRNA demonstrate that the transcript is detectable in 11/19 F₁ progeny whereas it is not detectable in 8/19. Plant inoculation assays show that all hybrid progeny lacking *Avr3a* transcripts are virulent towards *Rps3a*. Most progeny with detectable *Avr3a* transcripts are avirulent towards *Rps3a* but phenotypic penetrance of this trait appeared to vary with the level of expression of the *Avr3a* gene (Figure 3.11, 3.12, Table 3.5). The outcome of this experiment is unexpected and also differs from the test-cross results. The apparent 1:1 segregation of *Avr3a* expression in the F₁ progeny suggests a parental effect, such as imprinting or heterozygosity, might be influencing the outcome.

3.5 Maternal or paternal effects do not determine gene silencing of *Avr3a* in F₁ progeny from P6497 X ACR10

The inheritance of *Avr3a* in F₁ hybrids from the P6497 X ACR10 crosses was unusual and one of the possible causes of such non-Mendelian inheritance could be parent specific imprinting. Parental specific imprinting is the transcription and expression of only one allele from specific maternal or paternal parent due to the epigenetic marks (Pardo-Manuel de Villena et al., 2000). Therefore we identified the maternal parent of hybrids using mitochondrial DNA marker. For the 20 F₁ progeny from the P6497 X ACR10 crosses, results show that P6497 is the maternal parent for 11/20 whereas ACR10 is the maternal parent for 9/20 (Figure 3.13; Table 3.5). However, there are no apparent maternal or paternal effects on *Avr3a* gene silencing for progeny from this cross.

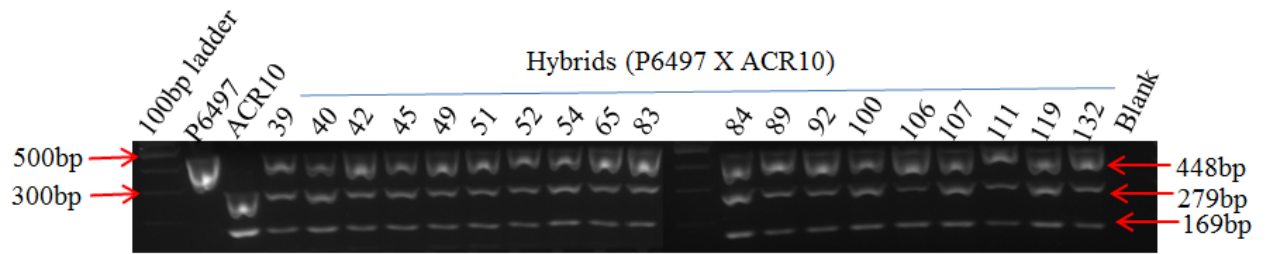


Figure 3.9 DNA marker analyses to determine hybrids from a cross of *P. sojae* strains P6497 X ACR10. The hybrids were determined using the CAPs marker *Scaf-29-M2* and *EcoRV* restriction enzyme digestion. Reverse primer *Scaf-29-M2-R* and forward primer *Scaf-29-M2-F* were used to perform the PCR reaction.

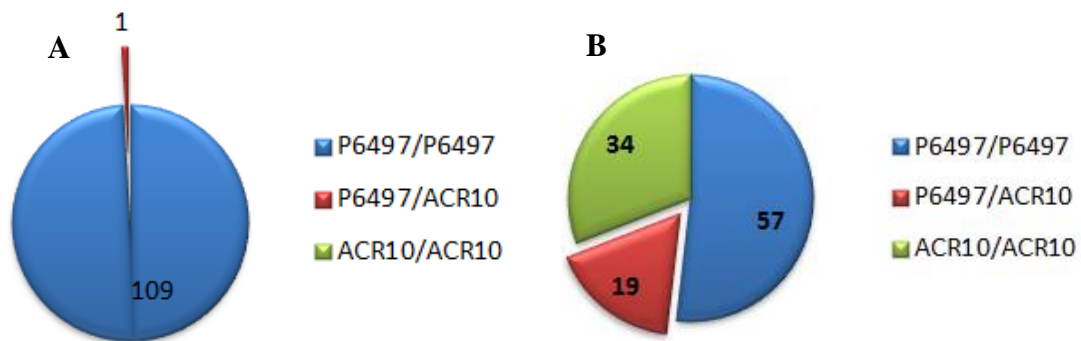


Figure 3.10 Self-fertilized and hybrid progeny from the cross of *P. sojae* strains P6497 X ACR10. (A) Results from the first attempt of P6497 X ACR10; only one hybrid was identified, most progeny arose from self-fertilized P6497 oospores. (B) results from the second attempt of P6497 X ACR10; 19 hybrids were identified, whereas 57 and 34 progeny arose from self-fertilization of P6497 and ACR10 oospores, respectively.



Figure 3.11 Photograph of virulence assay results of two different F₁ hybrids of P6497 X ACR10 cross. The F₁-111 hybrid was avirulent toward soybean plants carrying the *Rps3a* gene (L83-570) while F₁-92 was virulent; both F₁ cultures were virulent to control plants cv. Williams (*rps3a*).

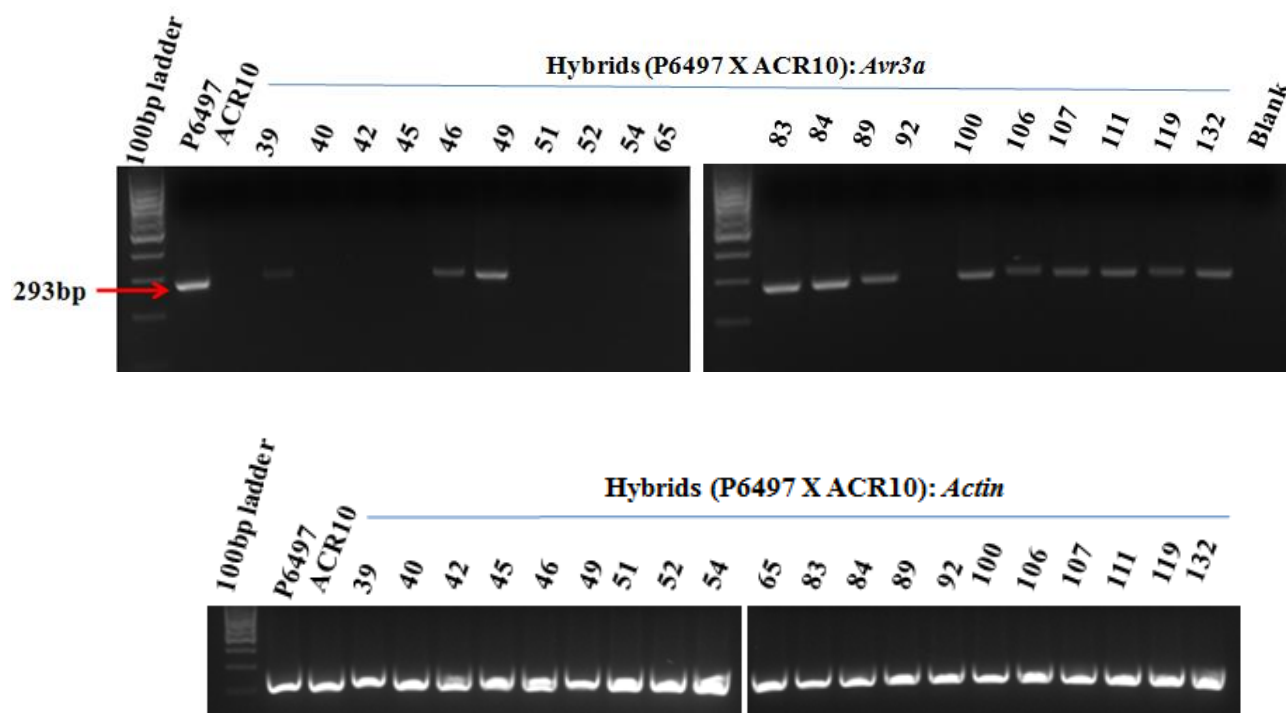


Figure 3.12 *Avr3a* gene expressions of F₁ hybrids of P6496 X ACR10 detected by RT-PCR. Gel red stained 2% agarose gel picture showing *Avr3a* gene expression and silencing in F₁ hybrids. P6497 and ACR10 are parental strains. The amplified product size of *Avr3a* gene is 293 bp. To perform RT-PCR, the *Avr3a* gene specific forward primer *Sp92-33F* and reverse primer *Sp92-325F* were used. *Phytophthora sojae Actin* gene was used as a control, to amplify *Actin*, forward primer *7-2g-MY(Actin A)-F* and reverse primer *7-2g-MY(Actin A)-R* was used.

Table 3.5 Scoring of parentage and virulence assay results of all F₁ hybrids (P6497 X ACR10) and their respective *Avr3a* gene expression pattern

P6497 X ACR10	Maternal parent	Virulence on L83-570 (<i>Rps3a</i>) ¹		Virulence on Williams (<i>rps3a</i>)		<i>Avr3a</i> mRNA transcript (RT-PCR)
		Number of dead plants /60	Assigned phenotype	Number of dead plants /60	Assigned phenotype	
39	P6497	59	V	60	V	Weak positive
40	ACR10	60	V	60	V	Negative
42	ACR10	60	V	60	V	Negative
45	P6497	56	V	60	V	Negative
49	P6497	18	A	53	V	Positive
51	ACR10	59	V	60	V	Negative
52	ACR10	54	V	56	V	Negative
54	ACR10	45	V	60	V	Negative
65	P6497	60	V	60	V	Negative
83	ACR10	17	A	60	V	Positive
84	P6497	0	A	60	V	Positive
89	ACR10	12	A	60	V	Positive
92	P6497	60	V	60	V	Negative
100	P6497	39	A'	60	V	Positive
106	P6497	0	A	60	V	Positive
107	P6497	2	A	60	V	Positive
111	ACR10	7	A	60	V	Positive
119	P6497	0	A	60	V	Positive
132	ACR10	5	A	60	V	Positive
46 (Trial 1)	P6497	4	A	60	V	Positive

¹Virulence assays consisted of two replicates of 30 plants each, for test (*Rps3a*) and control (*rps3a*) plants. V-virulent, A-avirulent, A' –incomplete virulent

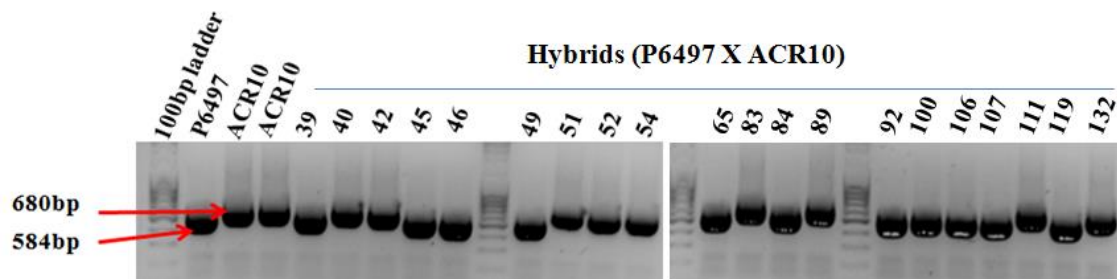


Figure 3.13 Mitochondrial DNA marker analyses of parental strains and F₁ hybrids from a cross of *P. sojae* strains P6497 X ACR10. Photograph of a gel red-stained 2% agarose gel showing PCR amplified products of a mitochondrial DNA fragment of *P. sojae* P6497 and ACR10, and F₁ progeny. To amplify the mitochondrial DNA segment, forward primer *mtI-P5008-F* and reverse primer *mtI-P5008-R* were used.

3.6 Self-fertilization of *P. sojae* strain P6497 and ACR10 produce true-breeding progeny for *Avr3a* virulence trait and gene expression

Parental heterozygosity is a possible explanation for 1:1 segregation ratios in F₁ progeny. To determine whether potential heterozygosity within the parental strains P6497 and ACR10 could account for the variation in virulence and *Avr3a* gene expression in the F₁ progeny, a total of 50 self-fertilized oospores were isolated from each of the strains. Out of total 50 progeny developed by self-fertilization of ACR10 X ACR10, 45/50 are virulent towards test plants (*Rps3a*) and lack detectable *Avr3a* transcripts, whereas 5/50 lack *Avr3a* transcripts and are avirulent towards test (*Rps3a*) and control (*rps3a*) soybean plants (Figure 3.14 A, 3.15 A). All of the self-fertilized progeny (50/50) developed by P6497 X P6497 are avirulent towards soybean *Rps3a* and possess detectable *Avr3a* transcripts (Figure 3.14 B, 3.15 B). Results show that the unusual inheritance of *Avr3a* expression is not due to the parental heterozygosity.

3.7 Segregation of virulence towards *Rps3a*, and *Avr3a* expression in F₂ progeny from P6497 X ACR10

To study the segregation pattern of the *Avr3a* virulence trait and gene expression in F₂ progeny from the cross P6497 X ACR10, independent F₂ populations were created by self-fertilization of six different F₁s. Three F₂ populations were from virulent F₁s lacking *Avr3a* mRNA transcripts, and three were from avirulent F₁s that express the *Avr3a* gene. A total of 150 F₂ progeny were isolated, including 75 for each of the two classes of F₁s. Results show that all 75 F₂ individuals arising from self-fertilization of virulent F₁s lacking *Avr3a* transcripts were virulent towards soybean carrying *Rps3a*

gene, and do not possess *Avr3a* mRNA transcripts (Figure 3.16 A and 3.17 B). By contrast, the F₂ populations from avirulent F₁ progeny segregate for virulent: avirulent phenotypes in a 1:3 ratio (Figure 3.16 B; Table 3.6). Results from RT-PCR analysis indicates that avirulent but not virulent F₂ progeny possess *Avr3a* mRNA transcripts, as expected (Figure 3.17 A).

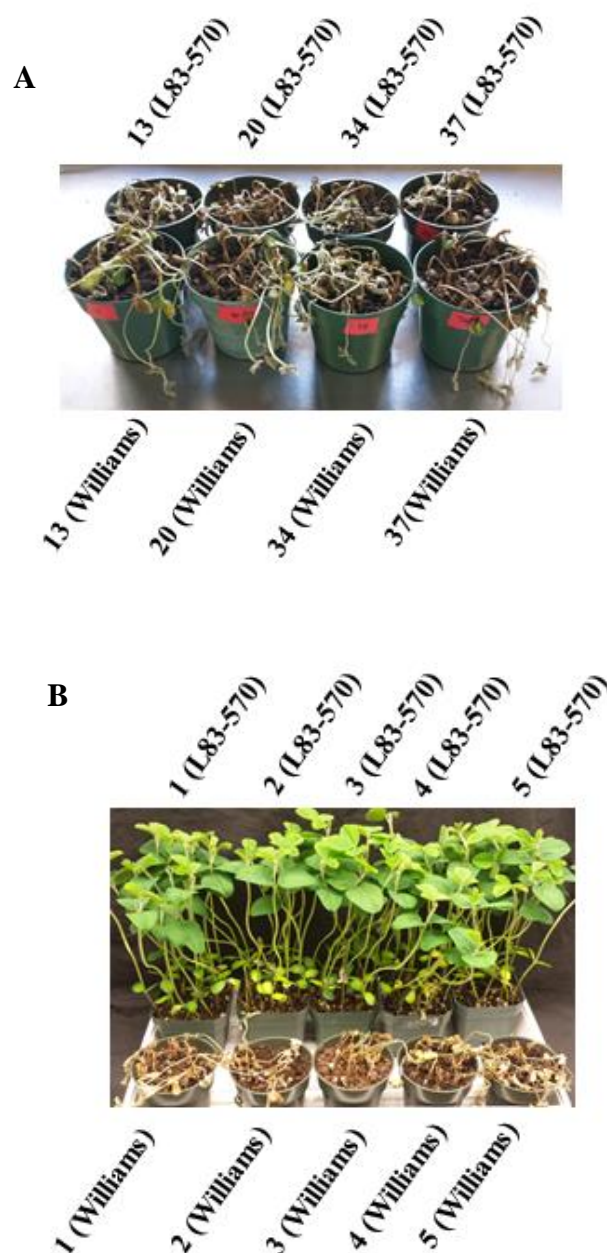


Figure 3.14 Representative virulence assays photographs of self-fertilized progeny.

(A) Photograph showing virulent assay of progeny generated from self-fertilization of *P. sojae* strains ACR10 X ACR10 towards L83-570 (*Rps3a*) and Williams (*rps3a*). (B) Photograph of disease outcomes of some progeny developed from self-fertilization of P6497 X P6497 towards L83-570 (*Rps3a*) and Williams (*rps3a*).

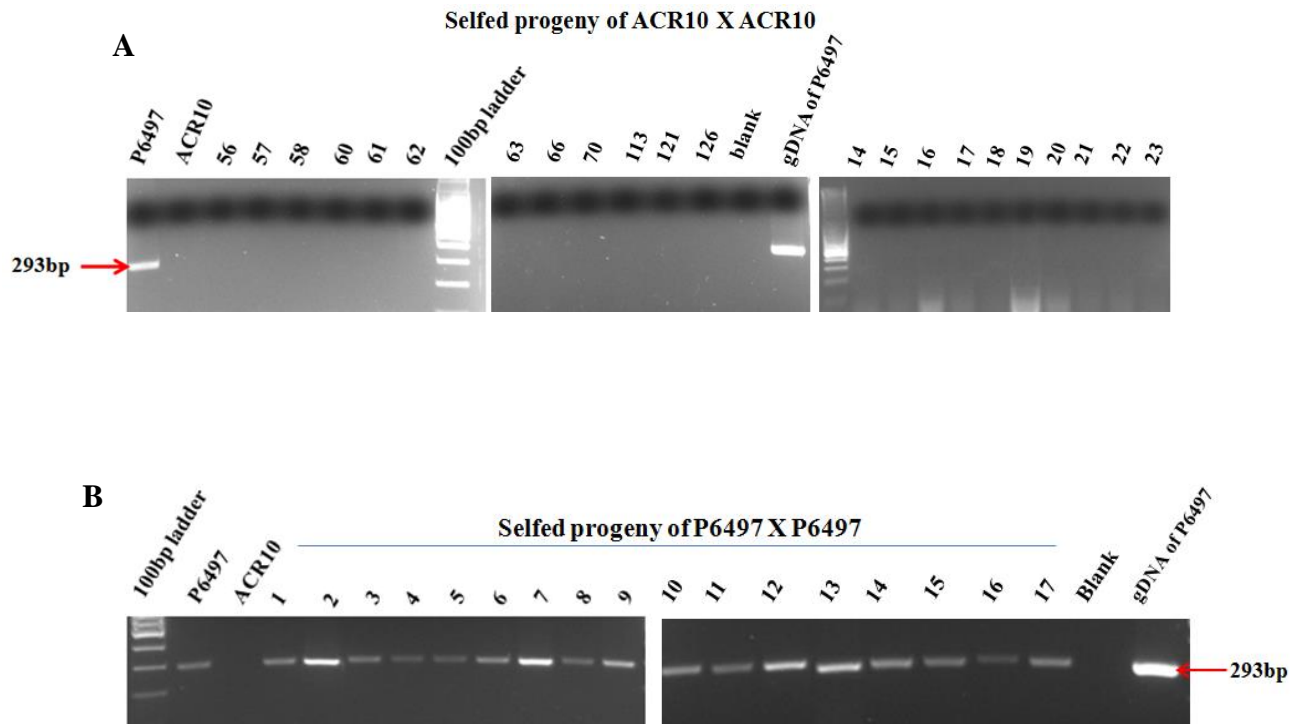


Figure 3.15 Parental *P. sojae* strains P6497 and ACR10 produce self-fertilized progeny that are true breeding with respect to the *Avr3a* virulence trait and *Avr3a* gene expression. (A) An RT-PCR analysis of *Avr3a* gene expression in parental strains and self-fertilized progeny from ACR10 ($Avr3a^{ACR10}/Avr3a^{ACR10}$). (B) An RT-PCR analysis of *Avr3a* gene expression in parental strains and self-fertilized progeny from P6497 ($Avr3a^{P6497}/Avr3a^{P6497}$) in gel red stained 1.5% agarose gel.



Figure 3.16 Representative virulence assays of F_2 progeny generated from self-fertilization of virulent and avirulent F_1 , from a cross of *P. sojae* strains P6497 X ACR10. (A) Photograph of disease outcomes for selected F_2 progeny from a virulent F_1 . All F_2 progeny isolated from virulent F_1 were virulent and killed all test (*Rps3a*) and control (*rps3a*) soybean plants. **(B)** Photograph of disease outcomes for selected F_2 progeny from an avirulent F_1 . The virulent: avirulent segregation ratio of F_2 progeny from avirulent F_1 is approximately 1:3.

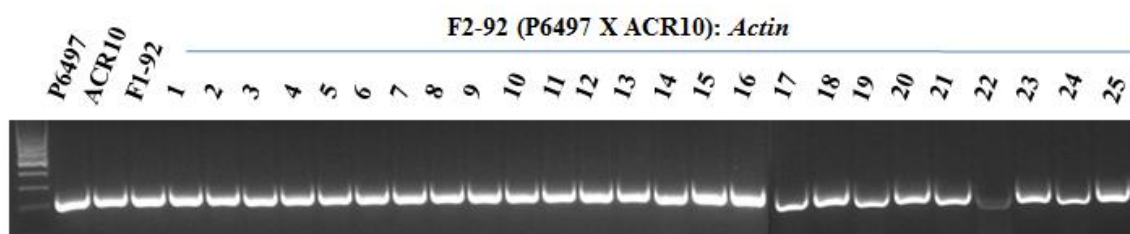
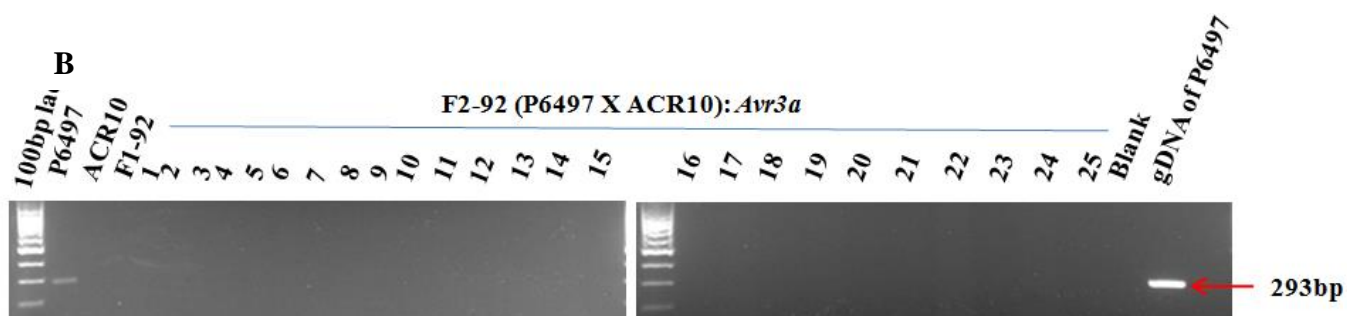
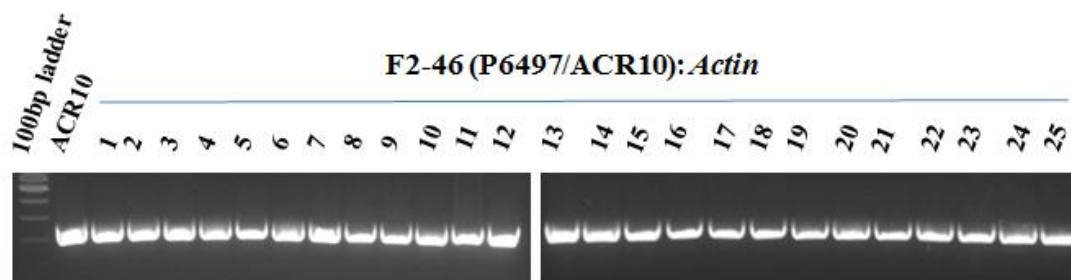
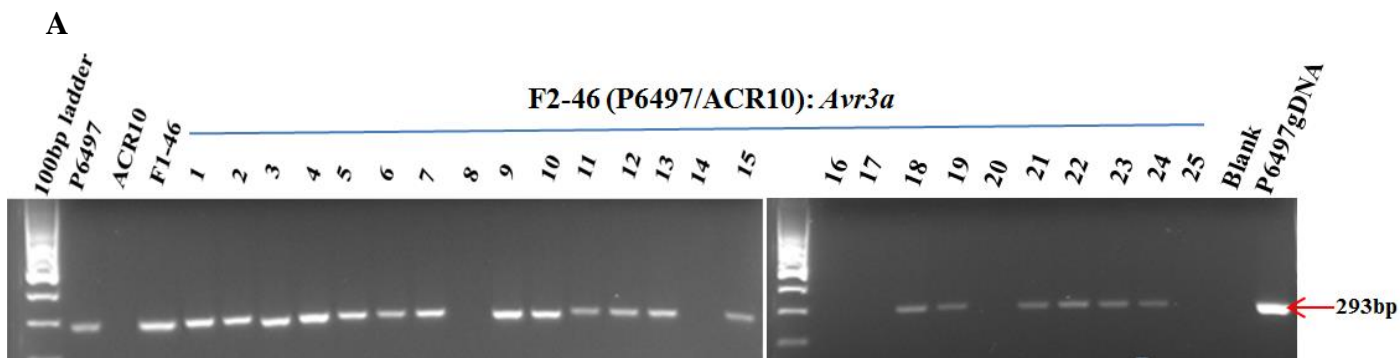


Figure 3.17 Analysis by RT-PCR of *Avr3a* gene expression among F₂ progeny from a cross of *P. sojae* strains P6497 X ACR10. Shown are photographs of 1.5% agarose gels, after staining with Gel Red fluorescent dye. *Phytophthora sojae Actin* gene was used as a control, to amplify *Actin*, forward primer 7-2g-MY(*Actin A*)-F and reverse primer 7-2g-MY(*Actin A*)-R was used. **(A)** Analysis of *Avr3a* and *Actin* gene expression of F₂ progeny derived from an avirulent F₁ (F₁-46) isolate. **(B)** Analysis of *Avr3a* and *Actin* gene expression in F₂ progeny derived from a virulent F₁ (F₁-92) isolate.

Table 3.6 Summary of results for virulence phenotype and *Avr3a* transcript analysis, for F₂ populations developed from avirulent F₁ (P6497 X ACR10).

F ₁ (P6497/ ACR10)	<i>Rps3a</i> Phenotype	<i>Avr3a</i> Transcript	Number of F ₂ s	Phenotype*		<i>P</i> - value from χ^2 analysis for F ₂ segregation
				Avirulent	Virulent	
F ₁ -46	A	+	25	19	6	0.91
F ₁ -111	A	+	25	21	4	0.30
F ₁ -119	A	+	25	16	9	0.20
Total			75	56	19	0.95

*F₂ progeny with avirulent phenotype were positive for *Avr3a* mRNA transcript but progeny with virulent phenotype were negative for *Avr3a* mRNA transcript, without exception.

Chapter 4: Discussion

4.1 Stable transgenerational gene silencing of *Avr3a* in *P. sojae*

Recent research has indicated that the variation of *Avr3a* mRNA transcript levels determines the virulence of *P. sojae* towards soybean plants carrying the resistance gene *Rps3a*. Strains that possess *Avr3a* mRNA transcripts are avirulent but strains lacking *Avr3a* mRNA transcripts are virulent towards soybean plants carrying *Rps3a* (Qutob et al., 2013; Qutob et al., 2009). It has also been found that crosses between *P. sojae* strains P7076 (avirulent phenotype; *Avr3a*^{P7076}/*Avr3a*^{P7076}) X ACR10 (virulent phenotype; *Avr3a*^{ACR10}/*Avr3a*^{ACR10}) results in transgenerational gene silencing of *Avr3a* and gain of virulence in all F₁ and F₂ progeny, despite normal segregation of the *Avr3a* gene itself (Qutob et al., 2013). To continue this research, homozygous F₂ progeny with the genotype *Avr3a*^{P7076}/*Avr3a*^{P7076} were self-fertilized to develop further progeny. Our results demonstrate that transgenerational gene silencing of *Avr3a* is meiotically stable in progeny from *P. sojae* strains P7076 X ACR10 to the F₅ generation. No *Avr3a* mRNA transcripts were detected in any of the progeny. The virulence assay results indicate that all the progeny retain virulence towards soybean cultivars carrying the *Rps3a* gene, with the exception of one outlier isolate in each of F₄ and F₅ progeny that suffers from a loss of pathogenicity. The loss of pathogenicity, indicated by a loss of virulence towards control plants lacking any known *Rps* genes, occurs spontaneously at low frequency in *P. sojae* cultures and progeny propagated in the laboratory (Na et al., 2014), so the two exceptions observed in this experiment are not unusual.

The stability of *Avr3a* silencing was tested because it is known from other examples of transgenerational or epigenetic phenomena that gene silencing can fade over generations (Jablonka and Raz, 2009; Rechavi et al., 2011). This could occur if an epigenetic factor required for silencing is not fully self-propagating. Meiosis is also a process that can re-set epigenetic control, especially for developmental or environmentally induced epigenetic changes that occur during somatic cell division (Iwasaki and Paszkowski, 2014; Kelly, 2014). Results from my experiments demonstrate that *Avr3a* gene silencing in progeny from *P. sojae* ACR10 X P7076 is stable through F₄ and F₅ progeny. Additionally, since the original cross was made in 2008, results show that *Avr3a* silencing is temporally stable over at least five years. Thus, if an epigenetic factor is responsible for *Avr3a* silencing, then this factor is exceptionally stable under the experimental conditions tested here.

Another reason to test stability of *Avr3a* gene silencing was to determine whether *Avr3a* expression could be reconstituted in F₄ or F₅ progeny. If transgenerational effects are simply the result of a multiple independent factors segregating in the cross, that are necessary for *Avr3a* expression but that are not recovered in the proper combination in the F₂ progeny, then reconstitution of *Avr3a* expression could occur in further generations. In simple epistatic silencing events, one would expect to find recombination and segregation in F₂ populations, and reconstitution of transcription of the *Avr3a* gene within some or many of the F₂ progeny (Scheid et al., 2002). This was not observed. However, if the P7076 strain is homozygous at three epistatic and unlinked loci that are required to maintain expression of the *Avr3a*, then the probability of reconstituting this genotype in the F₂ generation is quite low (~1.6%), and the chances decrease with

greater numbers of required epistatic loci. Thus, there is a chance that this simply did not occur in the original F₂ population of 139 individuals. The probability of reconstituting the genotype increases in F₃, F₄, and F₅ generations because self-fertilization and inbreeding lead to loss of heterozygosity (Xu et al., 2007). Results show that reconstitution of *Avr3a* expression did not occur in any of the F₄ or F₅ progeny, so it is not possible to conclude with certainty whether multiple epistatic loci are required. Nonetheless, a parsimonious interpretation of the outcome tends to discount the multiple-epistasis hypothesis. The scenario that *P. sojae* strain ACR10 is lacking multiple independent factors, each required in a homozygous condition specifically for *Avr3a* gene expression, is somewhat implausible.

A simpler hypothesis invoking epistatic loci to account for the silencing of *Avr3a* in progeny from *P. sojae* ACR10 X P7076 relies on another genetic phenomenon known as high frequency gene conversion, or loss of heterozygosity. This occurs in hybrid cultures generated from crossing different strains, and has been demonstrated in *P. sojae* and in other oomycete species such as *Pythium ultimum*, *P. parasitica*, *P. infestans*, and *P. cinnamomi* (Carter et al., 1999; Chamnanpant et al., 2001; Dobrowolski et al., 2002; Förster et al., 1994; Francis et al., 1994). For example, if *P. sojae* strains ACR10 and P7076 differ at an epistatic locus that is necessary for *Avr3a* gene expression or silencing, then gene conversion occurring at the epistatic locus in hybrid cultures could cause unusual inheritance patterns (Chamnanpant et al., 2001). However, this scenario would require that the hypothetical epistatic factor exclusively and spontaneously converts to the haplotype which results in *Avr3a* silencing. Although there are only a limited number of studies of gene conversion in oomycetes, the results tend to contrast with the observations

of *Avr3a* inheritance. First, loss of heterozygosity may show allele-specific bias but it is unusual for all hybrid progeny to exclusively convert to one allele. Second, loss of heterozygosity is time dependent, and tends to accumulate in hybrid cultures as they are clonally propagated, rather than occurring instantaneously (Blackburn et al., 2004). Therefore, the characteristics of *Avr3a* gene silencing in hybrid progeny differ from the known examples of loss of heterozygosity in oomycetes, but it is not possible to discount this hypothesis as an explanation.

4.2 Test-cross of *P. sojae* strains results in release of gene silencing

One of the goals of this research was to study whether the silenced alleles of *Avr3a* have an ability to silence the expressed alleles of other *P. sojae* strains, as was previously shown in the cross of *P. sojae* strains ACR10 X P7076. The outcome from the test cross of P6497 X F₁ (ACR10 X P7076) demonstrated that all progeny with the genotype *Avr3a*^{P6497}/*Avr3a*^{P7076} or *Avr3a*^{P6497}/*Avr3a*^{ACR10} expressed *Avr3a* mRNA transcripts. For the progeny with the genotype *Avr3a*^{P6497}/*Avr3a*^{P7076}, expression of both alleles was detected, and thus the silencing of the *Avr3a*^{P7076} allele was released by out-crossing. For the progeny with the genotype *Avr3a*^{P6497}/*Avr3a*^{ACR10}, it was not possible to determine allele specific expression, but it is clear that the *Avr3a*^{ACR10} allele does not have the capability to silence the *Avr3a*^{P6497} allele in this circumstance. The results differ from classical examples of paramutation, which is a phenomenon of gene-silencing where a paramutagenic allele has the ability to silence the paramutable allele, and paramutable alleles gain the ability to be paramutagenic (Chandler and Alleman, 2008; Pilu, 2011). However, the known examples of paramutation are diverse in their characteristics, so it

remains possible that paramutation and gene silencing of *Avr3a* may share underlying mechanistic features.

In the second replicate of the test cross, the phenotypic penetrance of the *Avr3a* avirulence trait was found to be incomplete in many of the progeny. A small percentage of test plants with the *Rps3a* gene were killed by inoculation with the progenies from the test cross. Furthermore, the *Avr3a* transcript level varied among the progeny and correlated well with the virulence profiles of the isolates. The results indicate that expression of the *Avr3a* gene is not fully restored in all of the progeny, and demonstrate a quantitative effect of *Avr3a* expression and avirulence towards the *Rps3a* resistance gene.

Although it is difficult to explain the variation of *Avr3a* transcript levels in the test cross progeny, the results clearly show that silencing is released by out-crossing, and that transgenerational inheritance does not occur. A possible explanation for this outcome is involvement of strain specific epistatic factors in *Avr3a* gene expression. As previously discussed, a hypothetical epistatic factor necessary for *Avr3a* expression or silencing can be invoked to explain the unusual inheritance patterns in progeny from the ACR10 X P7076 cross, if one assumes all the special conditions of gene conversion are met. Likewise, the release of gene silencing in the test cross hybrids could be due to the presence of strain specific epistatic factors in the *P. sojae* strain P6497 that regulate expression of *Avr3a* or that control epigenetic inheritance.

4.3 Inheritance of *Avr3a* in a cross of *P. sojae* strains P6497 X ACR10

Phytophthora sojae is a homothallic organism but nonetheless the success rate for obtaining hybrids in laboratory crosses depends on the parental strains (Förster et al., 1994). The first attempt to produce hybrid progeny from a cross of P6497 X ACR10 was not very successful, since 109/110 oospores resulted from self-fertilization of strain P6497. It is possible that the growth of strain P6497 simply out-competed strain ACR10 under the co-cultivation conditions used for cross. The single F₁ hybrid that was identified indicated that it is possible to produce sexual progeny from P6497 X ACR10. The second attempt to cross P6497 X ACR10 was more successful and 19 F₁ hybrids were identified from 110 oospores. Analysis of the 20 F₁ hybrids from both attempts showed that 12/20 produced detectable *Avr3a* mRNA transcripts whereas the 8/20 did not. Thus, the F₁ hybrids segregated in an approximate 1:1 ratio for *Avr3a* gene expression. Among the F₁ hybrids with detectable *Avr3a* mRNA, the level of expression appeared to be variable, but this was not rigorously tested by real-time, qRT-PCR.

The results from the P6497 X ACR10 cross show both differences and similarities to the results from the test cross of P6497 X F₁ (ACR10 X P7076). The test cross results were different because all 23 progeny with the heterozygous genotype *Avr3a*^{P6497}/*Avr3a*^{ACR10} were avirulent towards soybean *Rps3a* and expressed *Avr3a* transcripts, whereas these traits segregated in F₁ from the direct cross of P6497 X ACR10 that had the identical heterozygous genotype *Avr3a*^{P6497}/*Avr3a*^{ACR10}. Nonetheless, for the F₁ progeny that produced detectable *Avr3a* mRNA, from either cross, the expression level

appeared to be variable, and this influenced the phenotypic penetrance of the avirulence trait.

So far, epigenetic inheritance is widespread in filamentous fungi, and non-Mendelian segregation due to epigenetic inheritance was recorded since 1949 in *Neurospora* (Jablonka and Raz, 2009). The apparent 1:1 segregation of *Avr3a* expression in the F₁s from P6497 X ACR10 could not be explained by parent-of-origin effects or by heterozygosity of the parental strains. Possible parental-specific effects on *Avr3a* gene expression was tested by mitochondrial DNA analysis. The mitochondrion is a cytoplasmic organelle and the mitochondrial DNA is inherited in non-Mendelian manner, predominantly from the maternal parent (Sato and Sato, 2013). The results showed no correlation between maternal or paternal parentage and *Avr3a* expression. Therefore, I conclude that there are no parent-of-origin effects on *Avr3a* expression in *P. sojae* hybrids from P6497 X ACR10.

Another likely explanation for 1:1 segregation of traits in F₁ progeny is heterozygosity of the parental strains. We tested for heterozygosity of the parental strains by developing self-fertilized progeny from each. A true breeding organism always produces offspring with a similar phenotype for the particular trait under study, and can be assumed to be homozygous for the genes controlling that trait (Griffiths et al., 2000). The inheritance pattern in progeny from self-fertilization indicated that both parents, P6497 and ACR10, are true breeding for *Avr3a* expression. Therefore, parental heterozygosity cannot account for the unusual segregation pattern observed in the F₁ progeny from the outcross.

To further study the inheritance of *Avr3a* expression in progeny from the P6497 X ACR10 outcross, I developed F₂ populations from virulent and avirulent F₁ hybrids. The F₂ populations developed from virulent F₁ individuals were all virulent, whereas this trait segregated in F₂s derived from avirulent F₁ individuals. The overall segregation ratio of *Avr3a* expressing: *Avr3a* silenced F₂ progeny was 56:19, which fits a 3:1 ratio (chi-squared probability = 0.95). These results suggest that *Avr3a* expression is inherited as a simple dominant trait in the F₂ progeny that develop from F₁ hybrids expressing *Avr3a*.

A possible explanation to account for all of the results from the P6497 X ACR10 cross is that there is an epistatic factor necessary for the expression of *Avr3a* present in *P. sojae* strain P6497 but not in ACR10. In F₁ hybrid progeny, the epistatic factor is subject to spontaneous gene conversion causing loss of heterozygosity, resulting in the presence of *Avr3a*-silenced F₁ individuals that are true breeding. In F₁ progeny that express *Avr3a* transcripts, the epistatic factor remains in a heterozygous state, so that the F₂ progeny segregate in a 3:1 ratio for *Avr3a* expression.

Although there clearly must be epistatic factors that are necessary for *Avr3a* expression or silencing, invoking gene conversion or loss of heterozygosity of these factors to account for the inheritance of *Avr3a* expression is troublesome because special conditions must be assumed to fully explain the results. There is also evidence from other studies indicating that loss of heterozygosity cannot account for changes in *Avr* gene expression states in *P. sojae*. For example, the *P. sojae* *Avr1a* and *Avr1c* genes show apparent epiallelic variation in expression (Na et al., 2014). Sequence identical alleles of each, *Avr1a* and *Avr1c*, can produce mRNA transcripts or be silenced, in a strain-specific

manner. The inheritance of gene silencing has not been studied for *Avr1a* or *Avr1c*, but it is known that clonally propagated strains can spontaneously switch expression states for each of these genes (Na et al., 2014; Qutob et al., 2009). Loss of heterozygosity is not likely to underlie the *Avr1a* or *Avr1c* gene expression states in these studies because no hybridization events were involved. It has also been demonstrated that virulence towards *Rps1a* can be repeatedly lost and recovered in successive single zoospore isolates of *P. sojae*, an observation that cannot be explained by loss of heterozygosity (Rutherford and Ward, 1985).

Although the results presented in this thesis cannot be easily explained, they do indicate that there is strain-specific interplay between conventional- and epi-genetic variation in *P. sojae*. Support for this conclusion is provided by comparative genomic studies of *P. infestans* and its sister species (*Phytophthora ipomoeae*, *Phytophthora mirabilis*, and *Phytophthora phaseoli*) demonstrating that epigenetic regulators themselves can be highly polymorphic and show signs of positive selection (Raffaele et al., 2010). My results also discount a role for parental imprinting of *Avr3a* and provide a path forward for discovery of the factors that regulate the expression of this gene. For example, it should be feasible to map and identify such a factor in progeny from the P6497 X ACR10 cross, especially since *Avr3a* expression segregates as a simple dominant trait in the F₂ progeny sets from *Avr3a*-expressing F₁s. Success in this area would enable other questions to be addressed, such as the role of loss of heterozygosity in the inheritance of *Avr3a* expression states.

Chapter 5: Summary and Conclusion

The interaction of *P. sojae* *Avr* and soybean *Rps* genes determines the disease outcome in the interaction between these two organisms. Previously, it was shown that transgenerational gene silencing causes gain of virulence in outcrosses of *P. sojae* strain P7076 X ACR10 (Qutob et al., 2013). My work shows that transgenerational gene silencing of *Avr3a* allele of *P. sojae* is meiotically stable to the F₅ generation. Furthermore, by performing test-crosses of *P. sojae* strains P6497 X F₁ (ACR10 X P7076), I demonstrate that silenced alleles of *Avr3a*^{P7076} or *Avr3a*^{ACR10} do not have the ability to silence *Avr3a*^{P6497} alleles. This experiment also provided evidence for the release of gene silencing of *Avr3a*^{P7076}. In contrast, results from the cross of *P. sojae* strains P6497 X ACR10 show that the *Avr3a* gene is expressed in approximately half of the F₁ progeny (12/20) and silenced in nearly half of the progeny (8/20). Analysis of F₂ progeny developed from virulent F₁ isolates are virulent and do not express *Avr3a*, whereas F₂ progeny developed from avirulent F₁ isolates segregate for these traits in a 3:1 ratio. Finally, other experiments show that there are no apparent maternal- or paternal- effects on *Avr3a* expression in hybrids from ACR10 X P6497, and each of these parental strains are true breeding for *Avr3a* expression and virulence towards *Rps3a*.

The unexpected and unusual inheritance results obtained in my study indicate that there is strain-specific interplay between conventional- and epi-genetic variations in *P. sojae*. Therefore establishment and propagation of gene silencing is strain specific. The unusual segregation pattern of *Avr3a* expression in progeny from P6497 X ACR10 suggests that an epistatic factor necessary for the expression of *Avr3a* is present in *P. sojae* strain P6497 but not in ACR10. Gene conversion events in hybrid cultures could

explain unusual the inheritance patterns. Therefore mapping and identification of factors controlling *Avr3a* expression in progeny from P6497 X ACR10 cross is important. If the factor can be identified, it would be possible to test whether gene conversion and loss of heterozygosity can account for the unusual inheritance of *Avr3a* expression states.

P. sojae is one of the most devastating soybean pathogens that is spreading to soybean producing areas all over the world. The best way to control and manage this disease is to develop disease resistant soybean cultivars by plant breeding (Sugimoto et al., 2012). However, *P. sojae* strains can diversify and adapt to the host, and overcome *Rps* genes within 8-15 years of deployment (Lin et al., 2013). Therefore, identification and characterization of *Avr* genes is necessary to identify agronomically important and novel *Rps* genes and to continuously manage root rot disease of soybean.

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ABSTRACTS

Sirjana Shrestha, Kuflom Kuflu, Yun Zhang, B. Patrick Chapman, Mark Gijzen. Escape from host immunity by epigenetic gene silencing of virulence effectors. Abstract presented at the, XVI – International Congress on Molecular Plant-Microbe Interactions Program, 6-10 July, 2014, Rhodes, Greece.

Sirjana Shrestha, Kuflom Kuflu, Dinah Qutob, Mark Gijzen. Epigenetic Gene Silencing of Virulence Effectors in the plant Pathogen *Phytophthora*. Abstract presented in a poster session to the “International Plant and Animal Genome XXII” conference, San Diego, USA, Jan 10-15, 2014.